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# Genetics of bone mineral density and the associated influence on performance and stress fracture incidence in high-level endurance runners

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A thesis submitted in partial fulfilment of the requirements of the  
Manchester Metropolitan University for the degree of Doctor of  
Philosophy

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## Publications

The majority of the following part of this thesis is currently under second review:

Chapter 1: Herbert, A. J., Williams, A. G., Hennis, P. J., Erskine, R. M., Sale, C., Day, S. H. and Stebbings, G. K. 'The interactions of physical activity, exercise and genetics and their associations with bone mineral density: Implications for injury risk in elite athletes. *European Journal of Applied Physiology*.

The following have been published that are associated with aspects of this thesis:

Papadimitriou, I. D., Lockey, S. J., Voisin, S., **Herbert, A. J.**, Garton, F., Houweling, P. J., Cieszczyk, P., Maciejewska-Skrendo, A., Sawczuk, M. and Massidda, M. (2018) 'No association between ACTN3 R577X and ACE I/D polymorphisms and endurance running times in 698 Caucasian athletes.' *BMC Genomics*, 19(1) p. 13.

Stebbins, G. K., Williams, A. G., **Herbert, A. J.**, Lockey, S. J., Heffernan, S. M., Erskine, R. M., Morse, C. I. and Day, S. H. (2018) 'TTN genotype is associated with fascicle length and marathon running performance.' *Scandinavian Journal of Medicine & Science in Sports*, 28(2) pp. 400-406.

## Abstracts and conference proceedings:

The following have been presented at conferences that are associated with aspects of this thesis:

**Chapter 5: Herbert, A. J.**, Williams, A. G., Lockey, S. J., Erskine, R. M., Sale, C., Hennis, P. J., Antrobus, M. R., Brazier, J., Heffernan, S. M., Day, S. H., Stebbings, G. K. (2018). Bone mineral density and associated genetic variants in high-level Caucasian marathon runners. Proceedings of the 23<sup>rd</sup> Annual Congress of the European College of Sport Science (Dublin, ROI).

**Chapter 4: Herbert, A. J.**, Williams, A. G., Lockey, S. J., Erskine, R. M., Hennis, P. J., Sale, C., Day, S. H. and Stebbings, G. K. (2017). Associations of bone mineral density-related genes and marathon performance in elite European Caucasian marathon runners. BMJ Publishing Group Ltd and British Association of Sport and Exercise Medicine (Newcastle upon Tyne, UK).

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**Abbreviations:**

<i>AXIN1</i>	Axin 1
<i>AR</i>	Androgen receptor
<i>ATP</i>	Adenosine triphosphate
<i>BMD</i>	Bone mineral density
<i>BMI</i>	Body mass index
<i>BGLAP</i>	Bone gamma-carboxyglutamate protein
<i>CA++</i>	Intracellular calcium
<i>CTR</i>	Calcitonin receptor
<i>CT</i>	Computed tomography
<i>COMT</i>	Catechol-O-methyltransferase
<i>DKK</i>	Dickkopf
<i>DNA</i>	Deoxyribonucleic acid
<i>DXA</i>	Dual-energy x-ray absorptiometry
<i>ERC1</i>	ELKS/Rab6-interacting/CAST family member 1
<i>ERK1/2</i>	Extracellular signal-regulated kinase 1/2
<i>ESR1</i>	Oestrogen receptor 1
<i>FOSL1</i>	FOS like 1, AP-1 transcription factor subunit
<i>GWAS</i>	Genome-wide association study
<i>HWE</i>	Hardy-Weinberg equilibrium
<i>IAAF</i>	International association of athletics federations
<i>IGF1</i>	Insulin like growth factor 1
<i>IL1</i>	Interleukin 1
<i>IL1B</i>	Interleukin 1 beta
<i>IL6</i>	Interleukin 6
<i>IL18</i>	Interleukin 18
<i>JUNB</i>	JunB proto-oncogene, AP-1 transcription factor subunit
<i>LRP5</i>	LDL receptor-related protein 5
<i>MMPs</i>	Matrix metalloproteinases
<i>mL</i>	Millilitre
<i>MRI</i>	Magnetic resonance imaging
<i>NHANES</i>	National health and nutrition examination survey
<i>ng</i>	Nanogram

<i>NF-<math>\kappa</math>B</i>	Nuclear factor - $\kappa$ B translocation
<i>OPG</i>	Osteoprotegerin
<i>PA</i>	Physical activity
<i>PB</i>	Personal best
<i>PCR</i>	Polymerase chain reaction
<i>PGE<sub>2</sub></i>	Prostaglandin E <sub>2</sub>
<i>pQCT</i>	Peripheral quantitative computed tomography
<i>PTCH1</i>	Patched 1
<i>P2RX7</i>	Purinergic receptor P2X 7
<i>RANK</i>	Nuclear factor $\kappa$ -b
<i>RANKL</i>	Nuclear factor $\kappa$ -b ligand
<i>RED-S</i>	Relative energy deficiency in sport
<i>RPM</i>	Revolutions per minute
<i>SF</i>	Stress fracture
<i>SFEA</i>	Stress fracture elite athlete cohort
<i>SFRP4</i>	Secreted frizzled related protein 4
<i>SNP</i>	Single nucleotide polymorphism
<i>SOST</i>	Sclerostin
<i>STARD3NL</i>	StAR related lipid transfer domain containing 3 N-terminal like
<i>TGS</i>	Total genotype score
<i>TNFRSF11</i>	TNF receptor superfamily member
<i>TNFRSF11A</i>	TNF receptor superfamily member 11a
<i>TNFRSF11B</i>	TNF receptor superfamily member 11b
<i>VDR</i>	Vitamin D (1,25- dihydroxyvitamin D3) receptor
<i>WGS</i>	Whole genome sequencing
<i>WNT5B</i>	Wnt family member 5B
<i>WNT16</i>	Wnt family member 16

## Abstract

Low bone mineral density (BMD) is established as a primary predictor of osteoporotic risk but can also have substantial implications for athlete health and injury risk in the elite sporting environment. BMD is a highly multi-factorial phenotype influenced by physical activity and genetics. The exact contribution of these factors and the specific genetic variants association with BMD, particularly in athletic populations, has yet to be determined. Furthermore, few investigations have considered gene-environment interactions - in particular, whether specific genes may be sensitive to mechanical loading from physical activity and the outcome of such an interaction for BMD and potential stress fracture injury risk. Consequently, the overall aim of the current thesis was to investigate the genetic associations with BMD, stress fracture incidence and marathon performance in high-level endurance runners and compare these to a non-athlete cohort to explore genotype-physical activity interactions. BMD differences between endurance runners and non-athletes as well as between runners who had suffered a stress fracture in comparison to those who had not was observed. Additionally, *WNT16* rs3801387 and *BDNF-AS* rs6265 genotype-cohort interactions with BMD were observed whilst *P2RX7* rs3751143, *COL1A1* rs1800012 and *TNFRSF11A* rs3018362 genotype associations with BMD were also present. *WNT16* rs3801387, *COMT* rs4680 and *P2RX7* rs3751143 were associated with endurance runner status but no variants were associated with performance or stress fracture incidence. These results identify novel genetic associations with BMD and athlete status in an endurance running population as well as genotype-cohort interactions that influence BMD. In conclusion, there appears to be a genotype-dependent influence on athlete status as well as BMD, which may be influenced by physical activity level. Further research is needed to replicate the associations observed in comparable and different populations. Nonetheless, the work presented here has added to our understanding of the genetic associations with performance, BMD and stress fracture



incidence, which may have implications for exercise programme management and improving performance in high-level endurance runners.

# **Chapter 1:**

## **Literature review**

## 1.1 Bone

Bone consists of the fibrous protein collagen, mineralised with calcium phosphate/hydroxyapatite (Currey, 2013). The human skeleton consists of 80% cortical bone and 20% trabecular bone, although different anatomical sites portray different ratios. These two types of bone are formed in a lamellar pattern, in which collagen fibrils are placed in alternating orientations (Clarke, 2008). Both cortical and trabecular bone are composed of osteons, termed Haversian systems and packets, respectively.

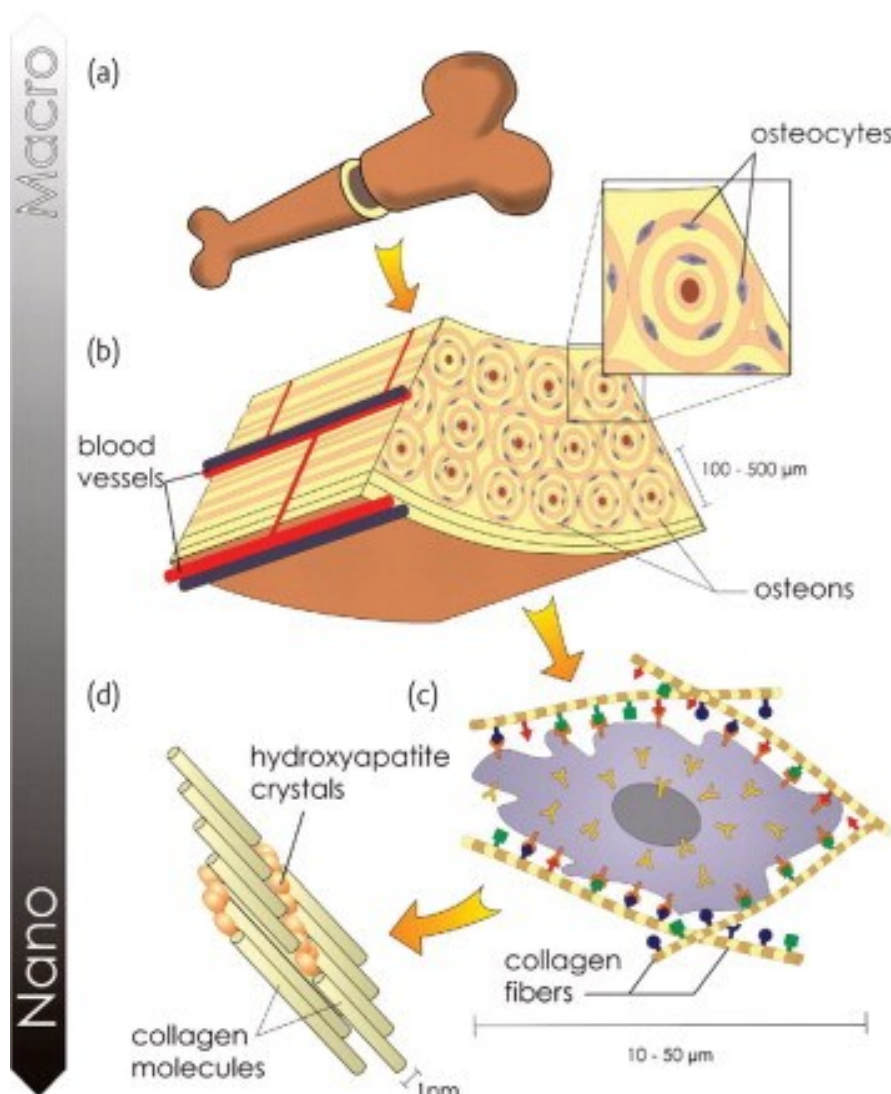


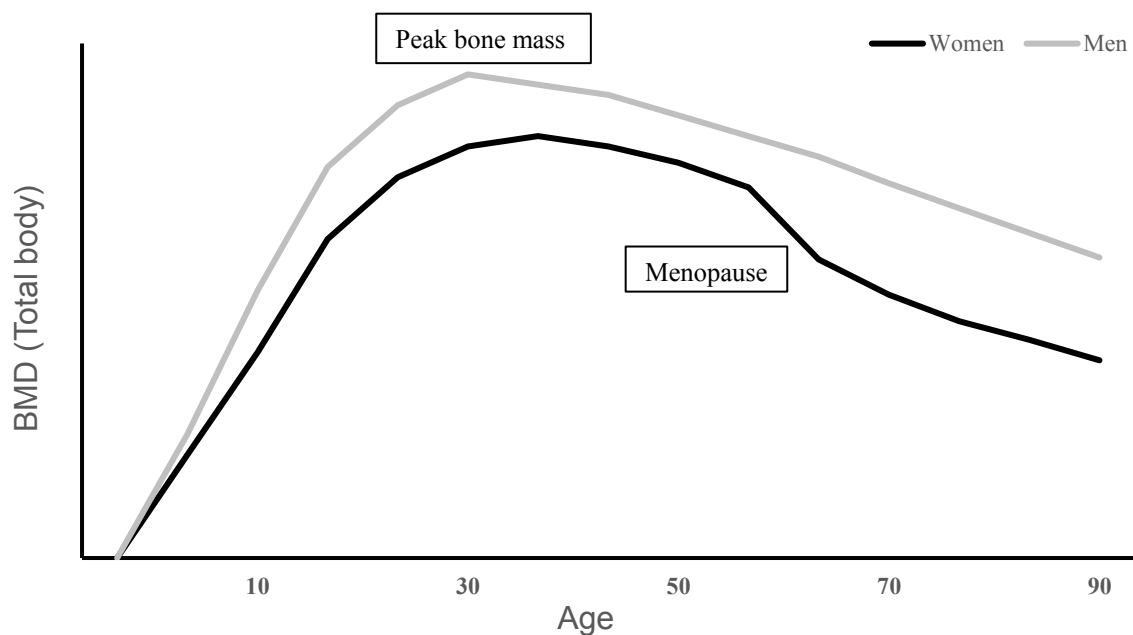
Figure 1.1: Structure of bone at cellular level. Taken from (Mitra et al., 2013).

### *1.1.2 Bone mineral density*

Peak bone mass is a function of bone size and volumetric bone mineral density (BMD) (Leonard and Bachrach, 2012) and thus, is the amount of bony tissue present following skeletal maturation, which can have a substantial influence on osteoporotic risk in later life (Bonjour et al., 1994). BMD is defined as the ratio of mass to the area or volume of bone, which is known as areal ( $\text{g}/\text{cm}^2$ ) or volumetric ( $\text{g}/\text{cm}^3$ ) BMD, depending upon the measurement methodology used (Ott et al., 1997). BMD is considered the primary predictor of osteoporotic fracture, although it is important to note other factors when assessing clinical risk (Cranney et al., 2007). BMD accounts for 60-65% of the variance in bone strength so other factors such as bone geometry, collagen properties as well as trabecular and cortical microarchitecture are also important determinants of bone strength (Schoenau et al., 2002; Fonseca et al., 2014; Cheung et al., 2016).

Bone mass is regulated by the activity of osteocytes in response to a number of stimuli, such as disuse, matrix damage or hormone deficiency (Atkins and Findlay, 2012) and the actions of osteoblasts and osteoclasts, which are important for bone formation and resorption. Disproportionate activity rates of these bone cells, for instance, greater net osteoclastic than osteoblastic activity, can cause bone loss, as observed in ageing (Martin and Sims, 2005). Approximately 85-95% of peak bone mass is attained around late adolescence (Henry et al., 2004; Walsh et al., 2009). After peak bone mass is reached, BMD loss occurs as we age (Figure 1.2) and the rate of loss plays an important role in bone health and the development of related conditions, such as osteoporosis (Hernandez et al., 2003). BMD deterioration varies between individuals as well as anatomical sites, with yearly rates of decline after the age of 25 years at the distal radius, distal tibia and lumbar spine reportedly 0.40%, 0.24% and 1.61%, in women and 0.38%, 0.40% and 0.84%, in men. Additionally, men and women experience 42% and 37% of trabecular bone loss as well as 15% and 6% of cortical

bone loss before the age of 50 years (Riggs et al., 2008). Similar to the ability to enhance peak BMD with lifestyle choices, it is possible to slow the inevitable decline in BMD with ageing using preventative measures via lifestyle modification. Some of these factors include not smoking (Law and Hackshaw, 1997), maintaining a healthy dietary intake (Darling et al., 2009) and relatively high physical activity level (Pluijm et al., 2001; Krall and Dawson-Hughes, 1993).



**Figure 1.2:** Schematic representation of typical age and sex-related loss of BMD in men and women.

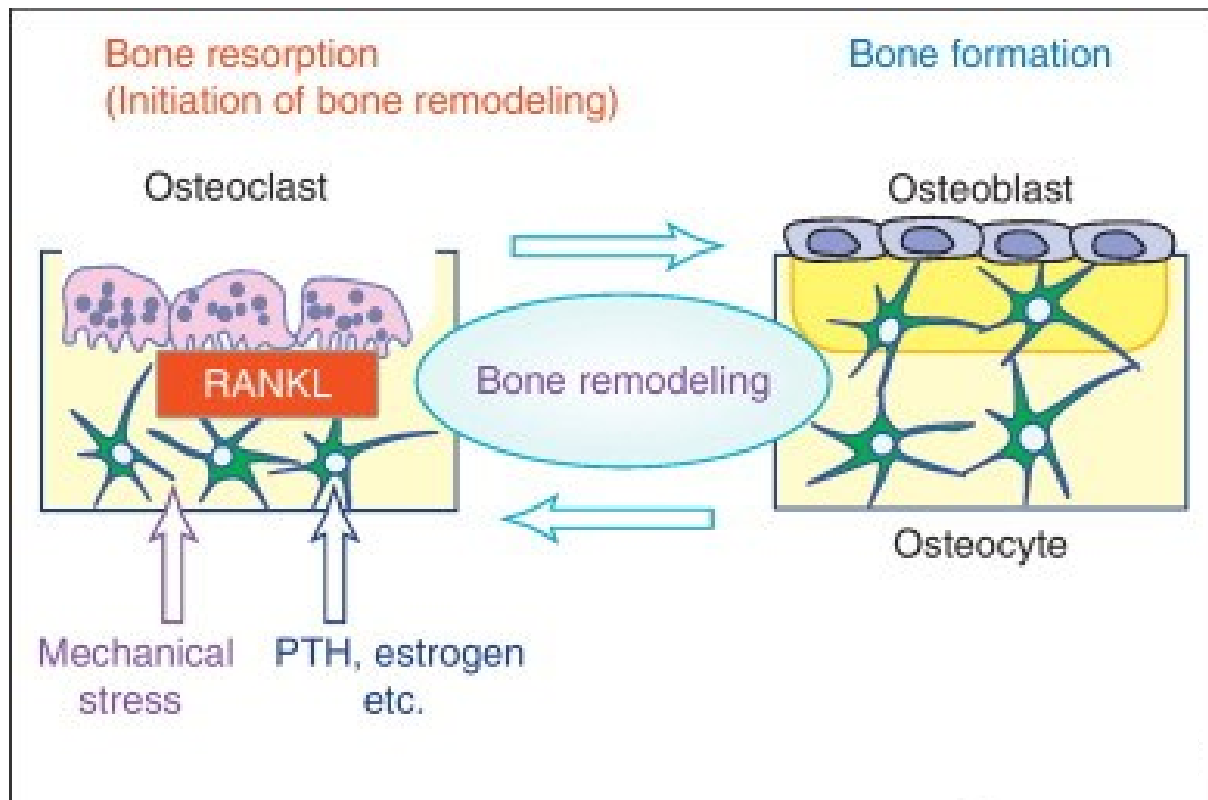
### 1.1.3 Bone mineral density and physical activity

Quantifying the relative contributions of physical activity and other determinants to BMD remains difficult. Exercise/physical activity reportedly accounts for up to 30% of the variability in total BMD (Table 1.1), emphasising that the contribution of physical activity to BMD remains unclear and requires further exploration across various population groups.

**Table 1.1:** Contribution of physical activity to BMD.

<b>Population</b>	<b>BMD determinant</b>	<b>Variability in BMD</b>	<b>Reference</b>
Icelandic women aged 16-20	Lean mass and physical exercise	30%	(Valdimarsson et al., 1999)
Men and women aged 20-25	Sports activities	10.4% - Men <1% - Women	(Neville et al., 2002)
Pre-menopausal women	Member of sports club Completing persistent weight-bearing activity in adulthood	5-19%	(Barnekow-Bergkvist et al., 2006)
European Caucasian men aged 65-80	High-impact unilateral training programme on one leg (EL) in comparison with the other leg (CL)	1.6% net gain in femoral neck between EL and CL	(Allison et al., 2015)
Men and women aged 20-54	Physical activity level	Active women and men had 2.7-4.6% and 1.9-3.0% higher BMD respectively than sedentary counterparts	(Morseth et al., 2010)
Men aged 17 - 20	Physical activity habits	10.1%	(Pettersson et al., 2010)

Initially proposed by Wolff's law and further developed by Frost's mechanostat theory, bone adapts or remodels in response to the forces or demands placed upon it (Frost, 1990). This mechanotransduction is completed through four steps: mechanocoupling, biochemical coupling, signal transmission and effector cell response (Duncan and Turner, 1995). Bone metabolism is regulated via specific pathways, such as the as nuclear factor  $\kappa$ -B/nuclear factor  $\kappa$ -B ligand/osteoprotegerin (RANK/RANKL/OPG), Wnt signalling and purinergic signalling pathways, through initiation of osteoblastic or osteoclastic activity (Tyrovola and Odont, 2015). Following physical activity, adenosine triphosphate (ATP) is released and osteocytes detect shape and volume changes to increase or decrease the liberation of these bone mediators, which consequently influences bone formation and resorption (Nakashima et al., 2011) as highlighted below (Figure 1.3).



**Figure 1.3:** Osteocytes modulation of bone metabolism through regulation of osteoblasts and osteoclasts (Nakashima et al., 2012).

This notion has been observed in numerous populations including children, adults and older adults, with those who complete a large volume of physical activity/exercise possessing greater BMD, strength and muscle mass (Chilibeck et al., 1995; Slemenda et al., 1991; Beck and Snow, 2003; Warburton et al., 2006). Corresponding with the higher BMD possessed by physically active or athletic populations, higher values for bone formation markers (e.g. osteocalcin, pyridinoline, deoxypyridinoline) have been reported in those undertaking higher training/loading/exercise in comparison with control populations (Maïmoun and Sultan, 2011).

The point in time when this physical activity occurs may also influence bone development and bone mass, potentially resulting in lifetime benefits for skeletal health (Gunter et al., 2012). Generally, weight-bearing activity in childhood has been shown to increase total BMD in adolescents and children (Weeks et al., 2008; Heidemann et al., 2013), as well as demonstrate a continued benefit into adulthood at key sites such

as the femoral neck and lumbar spine (Strope et al., 2015). Tveit et al. (2013) reported exercise-associated high BMD in 46 young male athletes (mean age = 22 years) was preserved three decades after retirement and cessation of high volumes of physical activity. Similarly, ex-professional baseball players in their ninth decade of life retained more than half of the throwing-related benefits in bone size and a third of the throwing-related benefits in bone strength observed in current professionals (Warden et al., 2014).

Some studies have suggested that activity completed in the pre-pubertal stage is the most favourable to instigate bone development, due to the elevated levels of growth hormone present at this time (Bass et al., 1998). Growing bone has an enhanced capability to respond to increased mechanical loading and thus initiate greater structural adaptations to this stimulus, compared to adult bone (Bass et al., 1998). This notion of an optimal period or “window of opportunity” for exercise-induced bone development could be important in improving bone health by maximising peak bone mass attainment during this time (Bass et al., 1998), and therefore, delaying the onset of age- or menopause-related osteoporosis (Santos et al., 2017). Despite this, Behringer et al. (2014) completed a meta-analysis and suggested that weight-bearing activities in childhood and adolescence had no significant influence on BMD in adulthood. The authors based their conclusion, however, on 27 studies out of a possible 109 completed before 2012 and suggests their findings might have been skewed as a result. Therefore, the overall consensus, as outlined by the National Osteoporosis Foundation’s recent position statement, is that the best evidence suggests a positive effect of physical activity during late childhood and pre-pubertal years and this is a key period for bone accretion (Weaver et al., 2016).

The ability to complete studies that are both longitudinal and valid, accounting for accurate measurement of activity (i.e. quantifying intensity in relation to the bone-



loading forces experienced) is extremely problematic. Many investigations have used self-report activity questionnaires rather than more direct measurements, such as via accelerometers or pedometers (Ondrak and Morgan, 2007). Self-report questionnaires rely upon recall and response bias, correlations between self-report and direct measurement of physical activity have been reported as low-to-moderate, ranging from -0.71 to 0.96 (Prince et al., 2008). Whilst accelerometers are capable of objectively quantifying activity level, this is still an estimation limited by validity, reliability and calibration concerns (Troiano et al., 2014), as well as being unable to provide direct measurement of the stimulus applied to any particular bone or the skeleton as a whole. Furthermore, there has been much methodological variance in studies exploring this topic, such as participant characteristics and sample size, the differing methods used to measure physical activity and types of physical activity/exercise completed in the training intervention. These factors make it difficult to draw conclusions on the exact influence of physical activity on BMD and may explain the large variability in the extent of the skeletal response to loading reported in intervention studies. For reviews on this topic see Warburton et al. (2006) and Ondrak and Morgan (2007).

Quantifying the optimum amount of physical activity for bone health is both difficult and complex when considering all of the potential confounding variables. Research has suggested the current US Department of Health and Human Services and UK Chief Medical Office physical activity guidelines do not allow maximisation of BMD potential (Whitfield et al., 2015). Additionally, the type of physical activity may also be important for optimising BMD. Habitual levels of high, but not moderate or light, physical activity was positively related to BMD in adolescents (Deere et al., 2012) as well as in older adults (Hannam et al., 2017). However, high impacts in adolescents were classed as  $>4.0g$  but only  $>1.5g$  in the older adults. Thus, the impact threshold to be bone protective is likely to be lower in older adults but higher g-forces may be required to

stimulate acquisition during peak attainment in childhood (Tobias, 2014), which adds further complexities to understanding the influence of physical activity on bone health. Therefore, due to the difficulty of quantifying physical activity and the large number of determinants of BMD, investigating the influence or association of physical activity on BMD is challenging. Using homogenous cohorts that are known to be undertaking similar amounts of physical activity, such as athletic populations, can somewhat alleviate this issue.

#### *1.1.4 Bone mineral density in athletic populations*

Physical activity can be defined as any movement implemented by skeletal muscle that results in energy expenditure, whereas exercise refers to physical activity that is planned, structured and repetitive with an aim to maintain or improve a physical fitness component (Caspersen et al., 1985). Therefore, athletic populations who complete large volumes of exercise, also tend to possess higher BMD and bone mass than non-athletic individuals via the loading adaptation mechanisms mentioned above (Chilibeck et al., 1995). However, the loading characteristics of different sports vary, thus the BMD of athletes partaking different sports or disciplines also varies, particularly between different anatomical sites (Mudd et al., 2007; Bennell et al., 1997). One of the earliest applied studies investigating BMD of athletes competing in different sports showed significantly higher total and site-specific BMD in volleyball players in comparison with gymnasts, swimmers and non-athletic controls, although the BMD of the gymnasts was significantly higher than the other two groups (Fehling et al., 1995). This emphasises that physical activity/exercise, which expresses higher impacts through increased strain rates and high peak-force loading characteristics, as can be expected of volleyball players, results in enhanced total or site-specific BMD as shown across of number of sports (Table 1.2).

**Table 1.2:** BMD variation across different sports.

<b>Population</b>	<b>Sport</b>	<b>BMD variation</b>	<b>Reference</b>
300 Norwegian female elite athletes (national level at senior or junior) 300 non-athletic controls	66 Sports	3-20% higher BMD than controls. 3-22% higher BMD in high impact sports compared to medium or low impact sports	(Torstveit and Sundgot-Borgen, 2005)
15 elite male athletes 15 non-athletic controls	Volleyball	14% and 24% higher BMD at the lumbar spine and femoral neck respectively in volleyball players in comparison with non-athletic controls	(Calbet et al., 1999)
14 state level female athletes 18 non-athletic controls	Netball	7.8%, 17.3% and 14% higher total body, hip and lumbar spine BMD in the netballers in comparison with the controls	(Chang et al., 2013)
50 male highly trained athletes 12 non-athletic controls	12 Judokas 14 Karate athletes 24 Water polo players	Control group total body BMD (1.27 g/cm <sup>2</sup> ) was significantly lower than the judo (1.40 g/cm <sup>2</sup> ) and karate (1.36 g/cm <sup>2</sup> ) group but no different to the water polo athletes (1.31 g/cm <sup>2</sup> )	(Andreoli et al., 2001)
59 competitive Finnish female athletes 25 physical active individuals 25 sedentary individuals	27 Dancers 18 Squash players 14 Speed skaters	Squash players had significantly higher BMD at the lumbar spine (13%), femoral neck (16.8%), proximal tibia (12.6%) and calcaneus (18.5%) in comparison with the sedentary group. Aerobic dancers also had significantly higher BMD at the loaded sites in comparison with the sedentary group, ranging from 5.3% to 13.5%	(Heinonen et al., 1995)
60 athletes 15 controls	15 Runners 15 Swimmers 15 Triathletes 15 Cyclists	Runners had significantly higher total body, femoral neck and leg BMD than controls and swimmers as well as higher leg BMD than cyclists.	(Duncan et al., 2002)

In endurance runners specifically, most studies have shown a higher BMD than control populations at the primary loading sites (tibia, femoral neck, calcaneus), although this is not always the case at other anatomical sites due to the influence of other variables, such as low energy availability. Although higher total-body (8.6%), lumbar spine (12.2%), femoral neck (9.7%) and leg (13.2%) BMD has been found in female adolescent runners in comparison to controls (Duncan et al., 2002), most studies have

reported lower or low (in relation to T- or Z-score) BMD in endurance runners at the non-loading sites (Hind et al., 2006; Barrack et al., 2008a; Barrack et al., 2008b; Pollock et al., 2010).

Endurance runners tend to possess lower BMD than athletes from other weight-bearing sports, such as volleyball or netball players, where forces applied to bone are more likely to be varied in magnitude and directions (Scofield and Hecht, 2012). Master athletes over the age of 65 years old who are still competing in running events have also been reported to possess higher BMD than non-active counterparts (Velez et al., 2008). Furthermore, former elite runners, soccer players and weightlifters have been shown to possess higher BMD than non-active controls as well as suffer osteoporotic hip fractures at a significantly older age (Kettunen et al., 2010). This emphasises the potential of BMD to be maintained and the importance of weight-bearing exercise in contributing to skeletal integrity in later life.

Studying athletes who experience extreme amounts of loading can somewhat compensate for the aforementioned limitations associated with quantifying physical activity. High-level athletes in weight-bearing sports are a unique population who generally experience extreme amounts of mechanical loading, which, although not a perfect solution, presents an attractive model for future research studies hoping to investigate the impact of exercise on BMD. Additionally, by selecting homogeneous athlete groups, who compete in the same event to a similar standard, it would be reasonable to assume these individuals undertake similar training regimes/volumes. For instance, Billat et al. (2001) reported high-level male marathon runners with a personal best of < 2 h 16 min ran an average weekly distance of 168 km ( $\pm$  20 km) and females with a personal best of < 2h 36 min completed 150 km ( $\pm$  17 km) on average. Although similar weekly running volume may be observed in endurance runners who are competing in similar competition distances, differences in loading variability

between runners exists. For example, substantial variation in maximum rearfoot pronation exists in elite female runners, which in turn may influence risk of Achilles tendonitis or shin splints (Williams, 2007), whilst level of ground reaction force may also contribute to running injuries (Hreljac, 2005). Consequently, other factors, such as those stated above alongside BMD, may influence risk and prevalence of injury in this population.

#### *1.1.5 Bone mineral density and stress fracture injury risk*

Despite the benefits of weight-bearing activity for BMD, at a high sporting level, too much activity to the point of overtraining can result in negative outcomes (Kuipers and Keizer, 1988). A stress fracture would be one such outcome and is defined as a partial or complete fracture of bone from repeated application of force lower than that required to fracture a bone in a single loading (Iwamoto and Takeda, 2003). Stress fracture injury occurs due to the repetitive mechanical loading that stimulates an incomplete remodelling response (Jones et al., 2002) and several factors are known to influence an individual's susceptibility to experiencing a stress fracture (Bennell et al., 1999). The exact pathophysiology of stress fracture is unknown but a proposed pathophysiological of stress fracture model in regards to altered/incomplete remodelling has been elucidated.

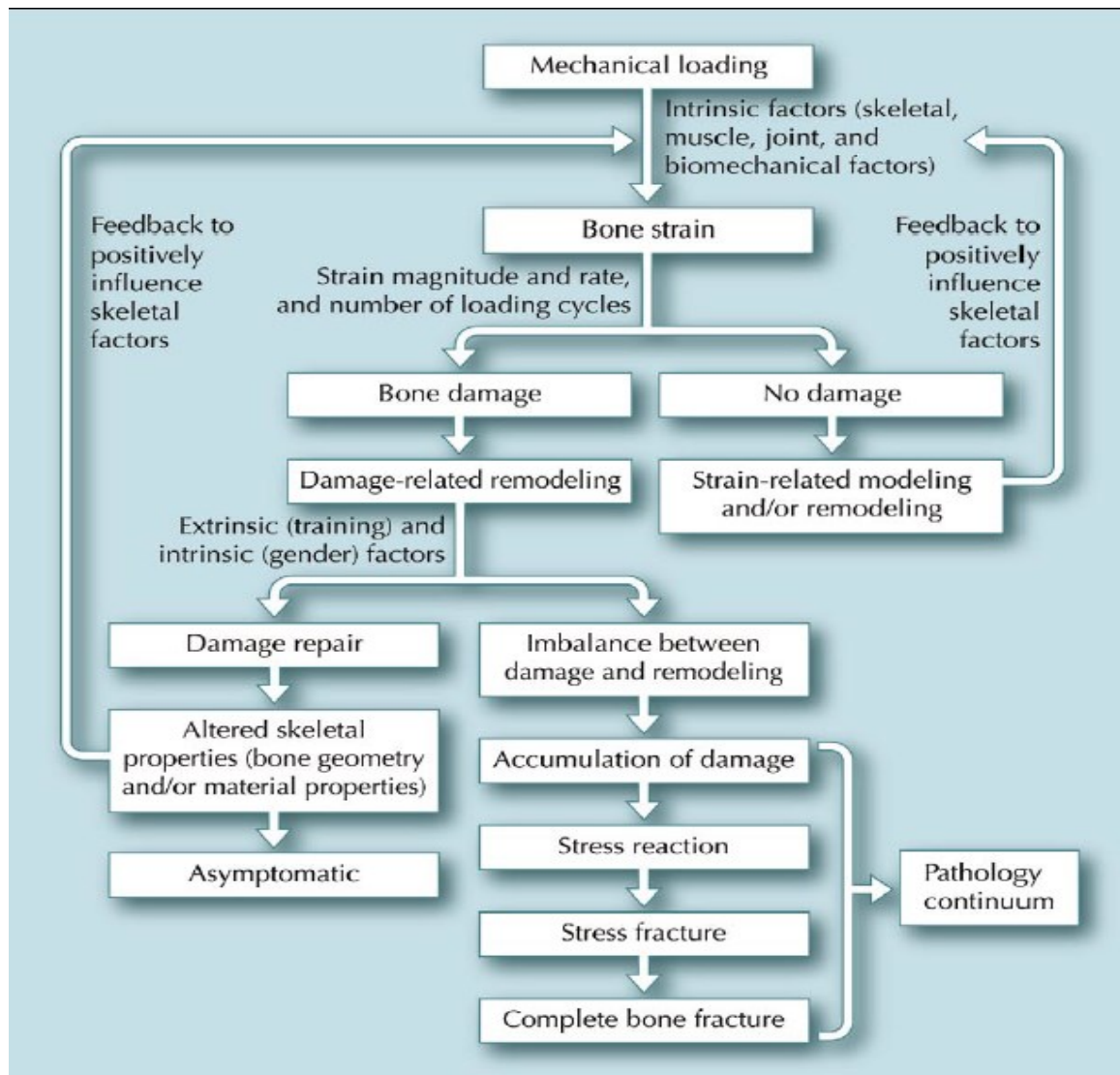


Figure 1.4: Proposed pathophysiological model of stress fracture. Figure taken from (Warden et al., 2006).

Stress fracture influencing/risk factors include biomechanical gait (Milner et al., 2006), bone size and mechanical properties (Tommasini et al., 2005), nutritional factors (Nieves et al., 2010), training volume and rapid increments in volume (Snyder et al., 2006), small musculature and low BMD (Beck et al., 2000).

Unsurprisingly, higher incidence of lower limb stress fractures is observed in endurance runners in comparison with non-athletic controls. Significant amounts of site-specific loading combined with other factors typical of this group, such as low energy availability, can result in lower BMD and a higher risk of fracture occurrence (Loucks, 2007). Stress fractures reportedly account for 50% of all injuries sustained by

runners and military recruits, with higher incidence observed in females (Milner et al., 2006). However, there is a lack of research on stress fractures in running populations (Wright et al., 2015). Furthermore, determining accurate prevalence is also difficult due to the problematic nature of defining stress fractures. Significant misdiagnosis will occur unless limited to radiography because other methods used lack sensitivity and specificity (Wright et al., 2015).

Although lower BMD has been observed at the foot in female athletes with a history of stress fracture, compared to those without, this was accompanied by lower lean mass, leg-length discrepancy and fewer menstrual cycles per year, which may be influential (Bennell et al., 1996). Contradictory findings regarding BMD association and stress fracture have been reported in the literature (Kelsey et al., 2007; Bennell et al., 1996; Crossley et al., 1999), which may be due to the differences in methodological study design. The lack of homogeneity and dissimilarity in both the participants used and the methodological design are likely to increase the inter-individual variability within the phenotype through differences in training characteristics and mechanical loading. Although a number of stress fracture risk factors for endurance runners have been proposed as mentioned above, female sex and previous history are the only two to have strong support for an association via meta-analysis (Wright et al., 2015). Consequently, further research investigating BMD in endurance runners is warranted. Investigating BMD, with a particular emphasis on injury, is undoubtedly important because stress fractures have substantial implications for athletes. For instance, Marathon world record holder, Paula Radcliffe, reportedly suffered a stress fracture 3 months before the Beijing 2008 Olympics, limiting her preparation for and performance at that competition. Furthermore, Ranson et al. (2010) reported 43% of the elite fast bowlers they investigated developed symptomatic acute lumbar stress fractures in a

two-year follow-up period and subsequently missed 169 days of cricket, per episode, on average.

If athletes are unable to complete their desired or required training volume due to injury, this could have substantial negative effects on their performance and success. Additionally, if an athlete knows they may be susceptible to injury this could be accounted for in their training programmes, by placing a greater emphasis on appropriate strengthening exercises and/or allowing longer rest periods between sessions. This valuable information for tailored training could then ultimately influence progression of athletes from amateur to elite or have implications for selection into high-level teams or sporting competitions. It is apparent that a substantial proportion of research in this area has been completed in military recruits (Wright et al., 2015). This is probably due to the ease of accessing large samples who undertake a quantifiable training load, as well as a desire to minimise waste of human and financial resources caused by injuries. However, it is difficult to directly extrapolate the findings of these military studies to high-level runners due to differences in the level of physical fitness, footwear and the loads carried whilst running between these groups. Despite the possibility of stress fractures, the positive benefits of physical activity/exercise on BMD in a broad population are evident. As discussed, there are a number of determinants influencing BMD but relatively little is known about the genetic influence on this phenotype and stress fractures, which could be pivotal for future understanding in both the sporting and public health domains.



## **1.2 Genetic influence on bone mineral density**

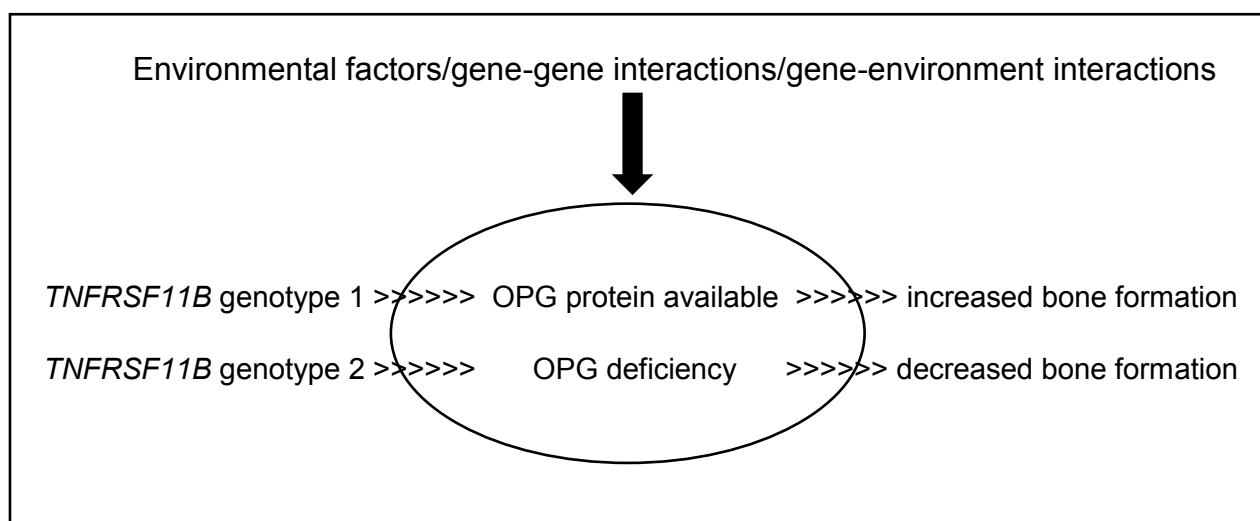
### *1.2.1 Genetic associations with bone mineral density*

Although BMD is a multi-factorial phenotype, heritability of BMD is suggested to be 50-85% depending upon anatomical location (Ralston and Uitterlinden, 2010). However, it must be emphasised that this proposed large genetic component is in a free-living population where most people will not complete extreme volumes of physical activity or be severely malnourished and thus, the influence of these other environmental factors on BMD will be reduced. Therefore, even a very substantial genetic contribution to BMD does not mean physical activity or other factors cannot notably affect an individual's BMD (as shown in section 1.1.3).

Due to this substantial genetic component, knowing the associated variants could be extremely beneficial for both functional research focus as well as application. For example, accuracy of fracture risk classification was improved by 7-10% at various sites in osteopenic patients by adding a genetic risk score from proposed common or rare variants associated with BMD and/or osteoporosis (Lee et al., 2014). In the future, this application might be utilised in athletic populations for risk stratification and injury prevention. However, implementing a genetic risk score with high-level athletes is currently difficult due to a lack of known candidate genes associated with BMD in athletic populations, which emphasises the need for replication of potential candidate genes and specific studies on particular populations, who may possess high or low BMD, or demonstrate specific lifestyle choices/habits that influence BMD.

Beginning in clinical populations, studies that selected candidate genes for association with BMD due to known biological function, such as Vitamin D (1,25- dihydroxyvitamin D3) receptor (*VDR*), insulin like growth factor 1 (*IGF1*) and oestrogen receptor 1 (*ESR1*) (Gong and Haynatzki, 2003), produced inconclusive findings. Candidate gene

selection can be based on the premise that the protein plays a role in regulating bone cell function or metabolism (Ralston and de Crombrughe, 2006), and the differing variants may affect bone mediators and consequently influence BMD. For example (as highlighted in Figure 1.5 below), the human TNF receptor superfamily member 11b (*TNFRSF11B*) gene encodes the protein osteoprotegerin (OPG), which regulates bone resorption by inhibiting differentiation and activation of osteoclasts. OPG-deficient mice have been found to develop early onset osteoporosis, and increased tissue mRNA expression has been observed in participants who possess specific haplotypes accompanied with reduced BMD, which may be due to increased expression resulting in stimulated osteoclast activity (Takács et al., 2010). This simplistic model forms the basis of genetic regulation on BMD but, in reality, the process is much more complex due to environmental factors and various kinds of interactions, which could have a substantial effect on gene expression and phenotype outcome. This potential impact of mechanical loading on gene expression can be understood by the substantial upregulation and downregulation of numerous genes following mechanical loading in rats (Mantila Roosa et al., 2011). Genes including FOS like 1, AP-1 transcription factor subunit (*FOSL1*) and JunB proto-oncogene, AP-1 transcription factor subunit (*JUNB*) were both upregulated within 4 hours after loading, whilst expression of Wnt/ $\beta$ -catenin signaling genes sclerostin (*SOST*) and secreted frizzled related protein 4 (*SFRP4*) was also altered at the synthetic phase of bone formation (Mantila Roosa et al., 2011). In the case of OPG, *in vitro* evidence demonstrated that compressive forces increased interleukin-6 (IL6) and prostaglandin (PG) E<sub>2</sub> production through activation of intracellular calcium/extracellular signal-regulated kinase 1/2 and nuclear factor  $\kappa$ B translocation (Ca<sup>++</sup>/ERK1/2/NF- $\kappa$ B) signalling pathways. This results in decreased osteoblast OPG expression (and a decreased OPG/RANKL ratio) and enhanced matrix metalloproteinases (MMPs) production, consequently increasing bone resorption (Sanchez et al., 2009).



**Figure 1.5:** *TNFRSF11B* genotype influence on OPG availability and subsequent bone formation with the potential of environmental and interaction effects.

Recent technological advances and large collaborations have seen a number of GWAS with BMD completed, which identified many more potential candidate genes and SNPs (Richards et al., 2012; Clark and Duncan, 2015). However, the most prominent study to date, a meta-analysis conducted by Estrada et al. (2012), identified 56 loci associated with BMD, osteoporosis and/or fracture that accounted for ~6% of the variation in BMD. Overall, more than 66 genetic loci have been associated with Dual-energy x-ray absorptiometry (DXA)-derived BMD via GWAS, as well as many others through candidate gene association studies, and this number continues to increase, emphasising the extremely polygenic nature of BMD (Golchin et al., 2016). A further 153 loci have been associated with BMD estimated by quantitative ultrasound of the heel (Kemp et al., 2017). A specific recent addition, for instance, is a locus harbouring the Patched 1 (*PTCH1*) gene in an Icelandic population (Styrkarsdottir et al., 2016). This rapid discovery rate of new candidate genes and the fact many previously discovered candidate genes have had little or no replication through further study, means only a very small number can be confidently suggested to have an association with BMD. Furthermore, the biological function or involvement with bone

metabolism of 30 of these has yet to be elucidated and only seven of the 66 have been associated in candidate gene studies previously or positively replicated afterwards (Hsu and Kiel, 2012), although some have received no further study as of yet. To have only seven candidate genes positively associated through both methods so far is surprising, considering almost 100 different loci have been associated with BMD via a candidate gene approach (Hsu and Kiel, 2012). Hsu and Kiel (2012) suggested a number of reasons why this may have occurred; firstly, false-negative findings due to the stringent level of statistical significance typically applied to GWAS data, or inadequate statistical power in some studies that were unable to replicate associations with modest effect sizes. On the other hand, false-positive findings of candidate gene association studies may have prevailed due to small sample sizes or publication bias (Munafo et al., 2004).

Additionally, strong gene-gene or gene-environment interactions could alter the number and identity of loci associated with BMD. This could apply to specific populations, such as athletes, due to the substantial influence of physical activity on BMD. Ultimately, this has resulted in few candidate genes emerging from GWAS and/or association studies that also have a known biological function relevant to bone. Therefore, further research using well-defined independent cohorts is needed to provide further evidence (Agueda et al., 2010). Clark and Duncan (2015) suggest greater use of “extreme cohorts” who might possess variants that have stronger associations with relevant phenotypes, which could include high-level athletes at one end of a continuum (as mentioned in section 1.1.4) and osteoporotic individuals at the other. This approach has been applied to BMD successfully in a study of postmenopausal women with extremely high or low BMD, where GWAS revealed six novel genetic associations (Duncan et al., 2011).

Studies so far have only elucidated a small fraction of BMD variance and thus, some of the unexplained heritability is likely due to a number of factors, including gene-environment interactions (Ackert-Bicknell and Karasik, 2013). Despite the substantial effect of physical activity/exercise on BMD, there has been little research regarding gene-physical activity interactions and its effects on BMD in athletic populations. Therefore, due to this limited amount of research, as well as the variance in sample size and participant characteristics, means it is difficult to evaluate the extent of the gene-physical activity interaction with BMD or propose any definitive candidate genes that interact with environmental factors in determining BMD. However, looking at this relationship using specific cohorts or populations is gathering momentum - for example, investigations exploring interactions with others phenotypes, including obesity, are now being conducted (Marti et al., 2008). As mentioned previously (section 1.1.4), athletes would be an excellent sample group to explore this interaction as they present an extreme cohort regarding exercise undertaken and BMD.

### *1.2.2 Gene-physical activity interactions on bone mineral density*

Mitchell et al. (2016) were the first to investigate the genetic influence on BMD and the relationship with physical activity using SNPs that had been associated with BMD using GWAS (Estrada et al., 2012). Analysis revealed physical activity interacted with ELKS/Rab6-interacting/CAST family member 1/Wnt family member 5B (*ERC1/WNT5B*) rs2887571 to influence bone mineral content in males and nominal interactions with physical activity were also observed with Wnt family member 16 (*WNT16*) rs3801387, axin 1 (*AXIN1*) rs9921222, *SOST* rs4792909 and stAR related lipid transfer domain containing 3 N-terminal like (*STARD3NL*) rs6959212. Sclerostin has a negative effect on bone formation by inhibiting canonical Wnt signalling in osteoblasts and also stimulates osteoclastic bone resorption by increasing the RANKL/OPG ratio (via enhanced RANKL expression) (Appelman-Dijkstra and

Papapoulos, 2016). Despite this strong influence on bone metabolism, conflicting results regarding *SOST* variants and association with BMD have been reported in the literature (Sharma et al., 2015). Additionally, serum sclerostin concentration has been positively correlated with lumbar spine, femoral neck and total hip BMD but no variants were associated with BMD or sclerostin concentration (He et al., 2014). It is important to note that children/young adults (age 5-19 yr) were the investigated cohort in the Mitchell et al. (2016) study. It is suggested some BMD-associated loci may exert age-specific effects (Medina-Gomez et al., 2012), and thus the findings cannot be generalised to other populations.

Interesting findings have also been reported in candidate gene association studies. Kiel et al. (2007) discovered two SNPs in the LDL receptor-related protein 5 (*LRP5*) gene associated with differences in BMD, which were dependent upon volume of physical activity completed. The TT genotype of both the rs3736228 and rs2396862 SNPs was associated with lower BMD in more physically active men, but with higher BMD in less physically active men. Thus, the authors hypothesised that the substitution of a C with a T allele in the rs3736228 SNP could alter LRP5-mediated Wnt signalling in the case that the catabolic signals induced from the mechanical loading prevail over anabolic signalling. This was also the case when expressing alleles as a haplotype *in vitro*, where the T allele was associated with a decreased response to canonical Wnt3a signalling in comparison to the C allele. Activation of Wnt/ $\beta$ -catenin (canonical) signaling increases the sensitivity of osteoblasts to mechanical loading, which can occur via Wnt binding to low-density lipoprotein receptor-related proteins 5 and 6 co-receptors (Robinson et al., 2006; Krishnan et al., 2006). This mediation of Wnt signaling via different *LRP5* variants can both enhance and decrease BMD (Ferrari et al., 2005). Loss-of-function mutations in *LRP5* are also responsible for low bone mass disorders, such as osteoporosis pseudoglioma, whereas gain-of-function mutations

have been suggested to cause high bone mass syndromes (Levasseur et al., 2005). Furthermore, *LRP5* variants, such as C135242T, have been associated with BMD variability in the general population (Koay et al., 2004) and ds2306862 in osteoporotic individuals (Mizuguchi et al., 2004), which highlights the strong influence *LRP5* may have on bone metabolism, particularly when considering a mechanical loading interaction.

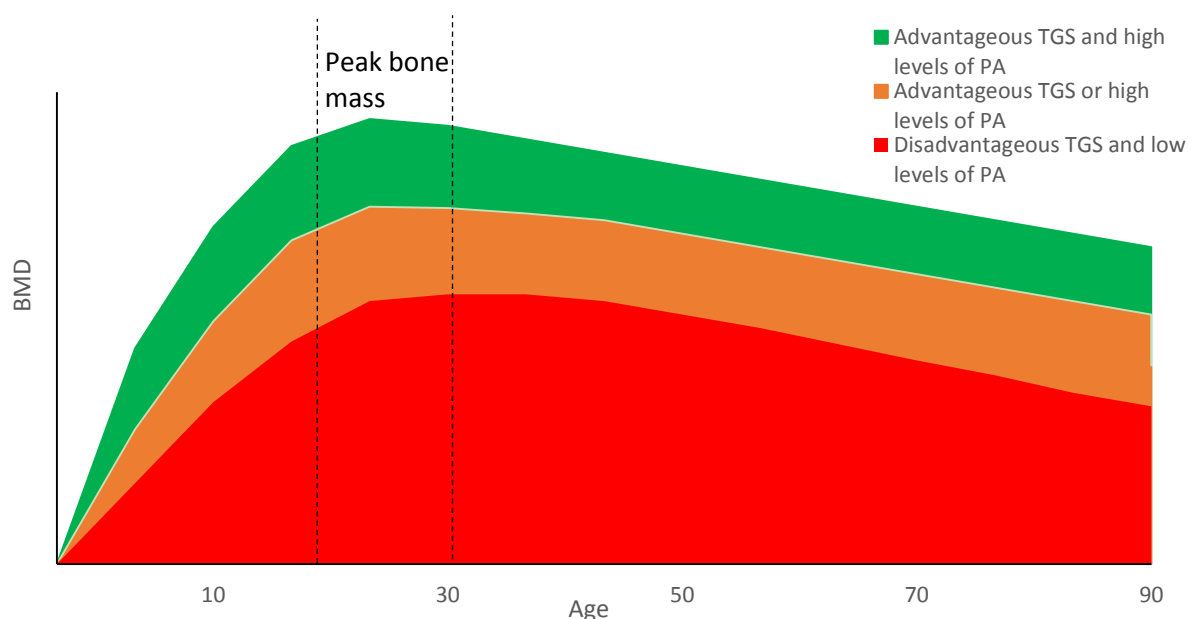
Similarly to some *LRP5* variants, the catechol-O-methyltransferase (*COMT*) val158met (rs4680) SNP has been reported to influence the association between physical activity and BMD, suggesting that certain variants may be particularly important for BMD in individuals with low physical activity levels. Higher total BMD was observed in individuals completing greater levels of physical activity (> 4 hours) compared to those undertaking lower activity (< 4 hours) for GA and AA (lower enzyme activity) but not GG (higher enzyme activity) genotypes (Lorentzon et al., 2007). Although lower BMD was observed in the lower enzyme activity group, estradiol serum levels were not. *COMT* catalyses the methylation of catechol oestrogens to methoxy oestrogens (inactive metabolites) and thus, lower *COMT* enzyme activity should result in less efficient inactivation of catechol oestrogens and higher BMD in these genotypes as has been shown in other studies (Eriksson et al., 2005). Therefore, a *COMT* genotype interaction may be present and the potential regulation of the BMD response to mechanical loading may be due to the involvement of oestrogen receptors as facilitators in a number of key pathways by which mechanical strain stimulates bone formation (Galea et al., 2013).

*IL6* is another potential candidate gene with a number of functional polymorphisms, suggested as candidates associated with BMD and/or osteoporosis. Meta-analysis revealed an association between the GG genotype in the *IL6* -174G/C (rs1800795) polymorphism and low BMD, as well as increased risk of osteoporosis, in a Caucasian

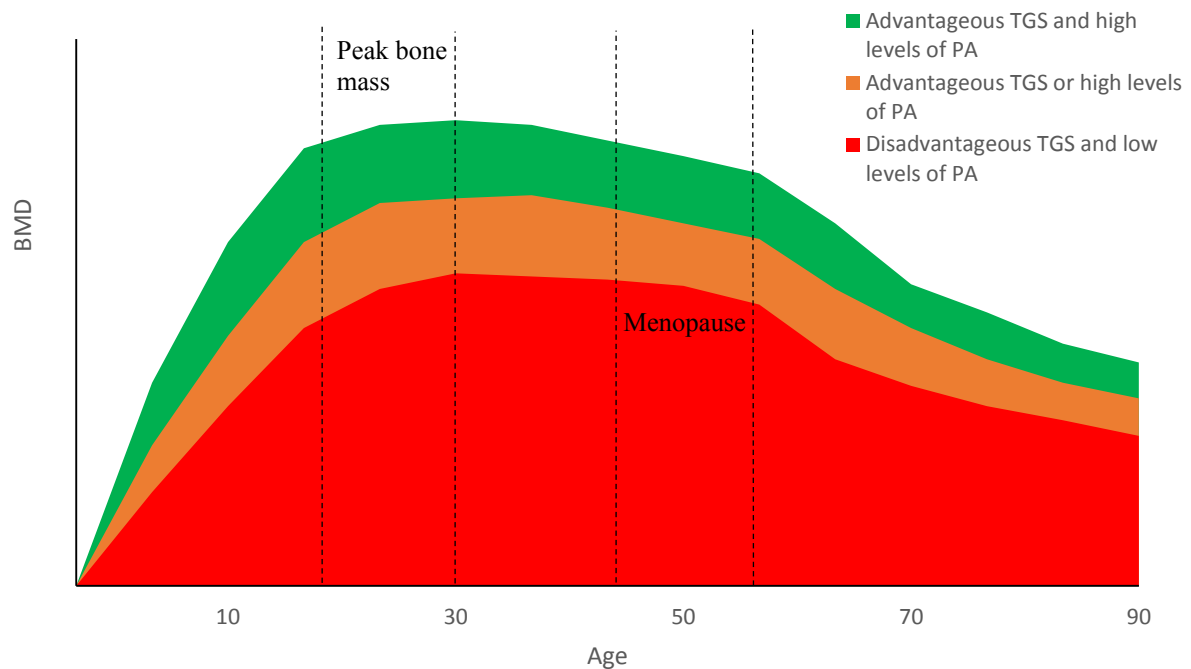
population (Ni et al., 2014). In the -634C/G (rs1800796) polymorphism, the CC genotype was significantly associated with greater BMD in Chinese pre-menarche girls who completed higher levels of physical activity (Li et al., 2008). Similarly, total body, lumbar spine and femoral neck BMD was lower in the GG genotype compared to the CC genotype by 0.03, 0.03 and 0.01 g/cm<sup>2</sup> respectively in an Asian population (n=3068) following meta-analysis (Yan et al., 2015). IL6 is primarily sourced in osteoblastic cells and increases interactions between osteoblasts and osteoclasts, thus stimulating bone resorption (Steeve et al., 2004). IL6 is suggested to indirectly stimulate osteoclastogenesis by increasing RANKL gene expression in osteoblasts (Bakker and Jaspers, 2015) and the G allele has been associated with elevated production and secretion of IL-6 *in vitro* (Kitamura et al., 2002). Therefore, the G allele and thus elevated IL6 may be disadvantageous for bone density. Although there are limitations regarding control of other BMD-influencing variables and various cohorts used in these studies, *IL6* remains interesting, particularly when analysing a possible relationship with physical activity. *In vitro* studies have suggested IL6 is produced by shear-loaded osteocytes and may influence bone mass by osteocytes reducing osteoblast activity via IL6-mediated intercellular signalling (Bakker et al., 2014). Elevated IL-6 serum concentrations have also been observed in trained marathon runners immediately post-race, with a positive correlation between IL-6 concentration and running intensity (Ostrowski et al., 2000). In longitudinal studies, serum IL6 concentration has been negatively associated with bone resorption and BMD in older adults, although the literature is somewhat conflicting (Ding et al., 2008). *IL6* demonstrates the possibility of strong gene-environment interactions and studies that do not control for physical activity risk erroneous findings and/or results that are only applicable to limited portions of the population.



Overall, completing weight-bearing physical activity has been shown to increase BMD as discussed in Section 1.1.3. The effect of potential gene-physical activity interactions on BMD across the lifespan, however, has yet to be determined. It could be hypothesised (Figure 1.6) that if an individual has a genetic profile/total genotype score (TGS) that is disadvantageous and completes low levels of weight-bearing physical activity (PA), they may be at risk for low BMD and potentially osteoporosis in later life (Disadvantageous TGS and low levels of PA). Those who may have a disadvantageous genetic predisposition, however, but complete sufficient weight-bearing activity to produce a substantial osteogenic response may be able to combat their negative genetic predisposition resulting in increased BMD, as evidenced in children (Mitchell et al., 2016) (Advantageous TGS or high levels of PA). Similarly, those who do not complete suitable levels of activity but possess an advantageous genetic profile, may also present with moderate BMD (Advantageous TGS or high levels of PA). Those with an advantageous genetic profile who also complete large volumes of weight-bearing physical activity are likely to have the highest BMD (Advantageous TGS and high levels of PA), which could be induced from a gene-physical activity interaction.

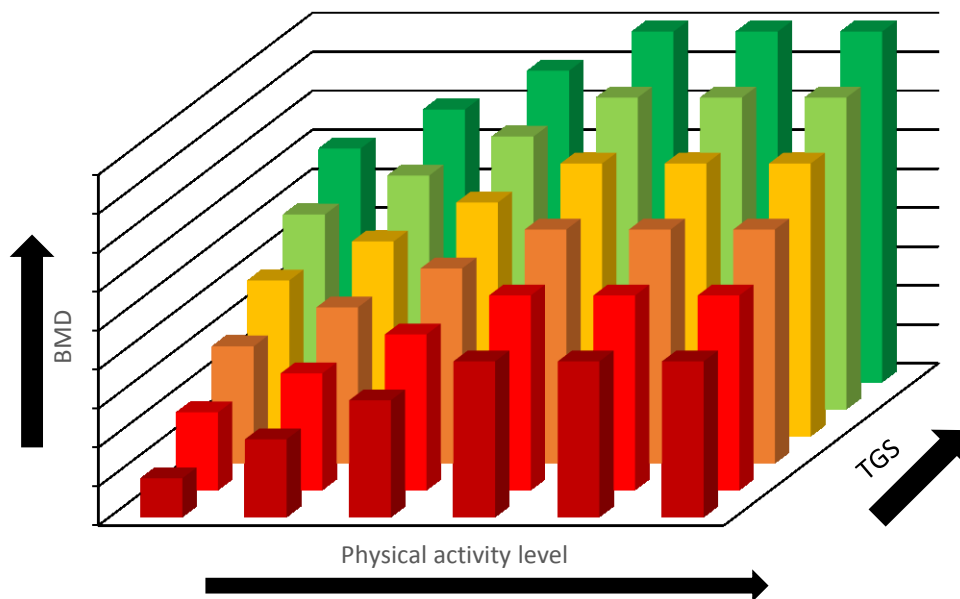


**Figure 1.6:** Schematic representation of typical age and sex-related loss of BMD in men and the effect of physical activity and genetics.



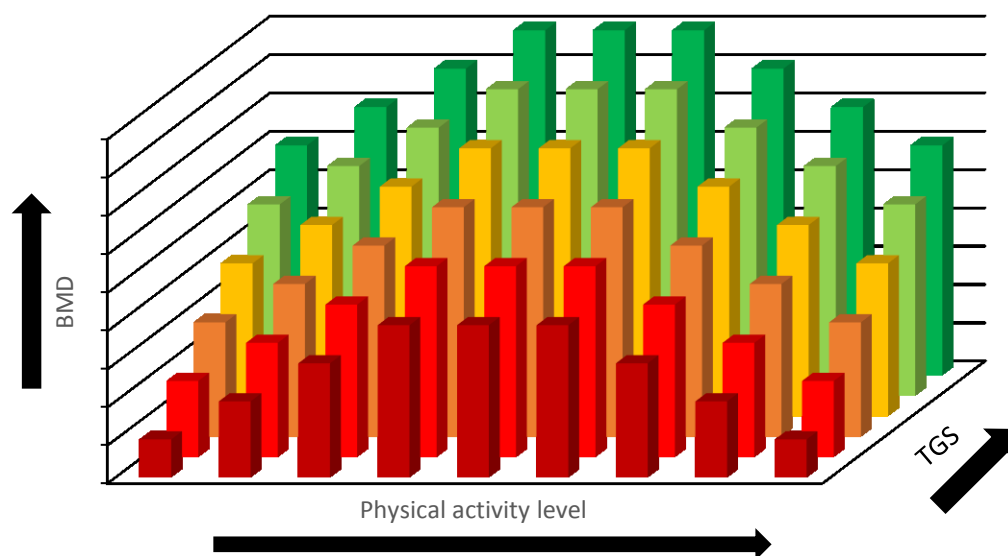
**Figure 1.7:** Schematic representation of typical age and sex-related loss of BMD in women and the effect of physical activity and genetics.

In the case of a gene-physical activity interaction, a hypothetical relationship between genetics, physical activity and the resultant BMD is presented below (Figure 1.7). Each bar represents a different individual and a hypothetical scenario for BMD ranging from a low BMD to a high BMD (the bar colour indicates BMD at any given level of physical activity in Figures 1.6 and 1.7). BMD is dependent on both genetics and physical activity level, so as physical activity level increases, BMD is enhanced for every individual regardless of their BMD before this increase in physical activity occurred. The magnitude of increase in BMD, and maximum BMD level attained, however, is under the influence of genetics (Ralston and Uitterlinden, 2010). Consequently, those with a more advantageous genetic predisposition, indicated by a higher TGS, combined with a higher volume of mechanical loading are more likely to reach a higher BMD than those with a disadvantageous genetic predisposition and/or a lower volume of mechanical loading, assuming all else is equal.



**Figure 1.8:** Schematic hypothetical representation of the BMD outcome for different individuals representing variable genetic profiles (TGS) and levels of physical activity.

It is possible, however, that a linear relationship between physical activity dose and BMD response does not exist at the extremes of physical activity (Figure 1.8). NHANES (National Health and Nutrition Examination Survey) data has previously demonstrated that BMD did not differ between males who reported completing 4-6 times more physical activity than the recommended guidelines (Whitfield et al., 2015). The physical activity and BMD relationship is still poorly understood and in the case of endurance runners, overtraining can negatively affect BMD (Figure 1.9) due to the associated influence of energy availability. Other factors such as the type of activity and dietary intake, however, are also important in regards to the bone adaptation as discussed in Section 2.2 and would consequently affect this relationship.

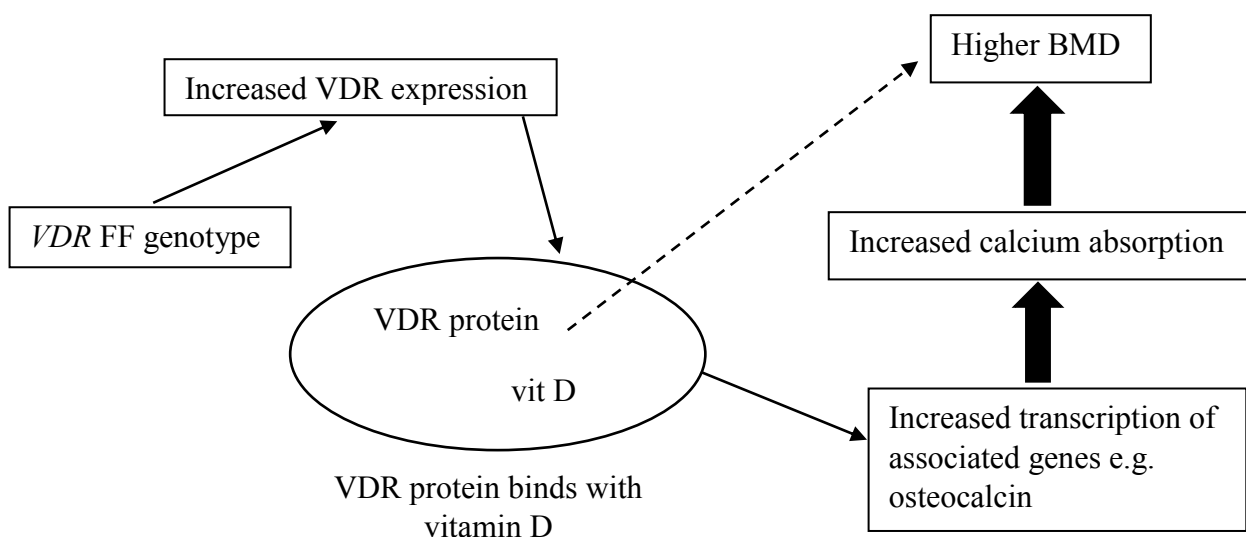


**Figure 1.9:** Schematic hypothetical representation of the BMD outcome for different individuals (e.g. endurance runners) representing variable genetic profiles (TGS) and levels of physical activity.

### 1.2.3 Genetic associations with bone mineral density in athletic populations

In 212 young males, significantly higher total BMD in 84 weight-bearing athletes than 80 controls was observed in the FF (7.7%) and Ff (6.9%) but not ff (1.8%) genotypes of the *VDR* FokI rs2228570 polymorphism, whilst significantly lower total BMD was only observed in the FF (-4.5%) genotype when comparing 48 swimmers with a control group (Nakamura et al., 2002a). This suggests that individuals with the FF genotype may be more responsive to mechanical loading, resulting in greater BMD when that environmental factor is prominent. This notion was further reinforced in 44 Japanese track and field athletes, where higher bone volume was expressed in those with the FF genotype, but not in those with the Ff genotype (Nakamura et al., 2002b). This particular polymorphism, FokI (rs2228570), exhibits a C to T transition that creates an upstream initiation codon, leading to the production of VDR proteins that are three more amino acids in length. The F allele codes for the absence of the restriction, whilst the f allele codes for the presence of the initiation codon, which leads to the longer amino acid length (Gross, 1996; Ames et al., 1999). It is suggested that the F variant

shows greater transactivation (protein expression) than the f variant and this increased biological activity (and associated increased intestinal absorption of calcium) could explain why higher BMD has been reported in those with the FF genotype (Arai et al., 1997; Colin et al., 2000; Uitterlinden et al., 2004; Ames et al., 1999) as detailed below (Figure 1.10). *VDR* controls the transcription of other genes including bone gamma-carboxyglutamate protein/osteocalcin (*BGLAP*) that are instrumental for this calcium absorption and bone formation (Moran et al., 2014). A direct effect of osteoblastic/osteocytic *VDR* signalling on bone remodelling has also been proposed, although specific understanding of this notion is still lacking and largely depends on calcium balance (Lieben and Carmeliet, 2013).



**Figure 1.10:** *VDR* rs2228570 FF genotype and the associated pathways leading to enhanced BMD.

The potential association of *VDR* with BMD and/or fracture has also been supported across a number of different SNPs (rs1544410, rs7975232 and rs731236) in various cohorts, such as pre and postmenopausal women (Riggs et al., 1995; Horst-Sikorska et al., 2007; Ji et al., 2010; Marozik et al., 2013). However, contradictory results have also been reported across these cohorts (Horst-Sikorska et al., 2013; Moran et al., 2015; Castelán-Martínez et al., 2015; Dabirnia et al., 2016). The highly conflicting

nature of the findings may be due to not adjusting for covariates such as body mass index (BMI) as well as the different ethnic groups, sample sizes and study designs utilised (Xu et al., 2005).

A recent study of 99 elite academy footballers found a number of SNPs associated with bone phenotypes (trabecular density, cortical thickness and cross sectional area) using peripheral quantitative computed tomography (pQCT) analysis. However, these associations were only observed before, but not after, a 12-week period of increased football training volume and thus, no genotype by time interactions were present. These variants included *SOST* rs1877632, Purinergic receptor P2X 7 (*P2RX7*) rs1718119, *P2RX7* rs3751143 as well as *TNFRSF11A*, *TNFSF11* and *TNFRSF11B* SNPs rs9594738, rs1021188 and rs9594759 (Varley et al., 2018). Although no genotype by time interactions were observed for the SNPs analysed in this investigation, other candidate genes could be sensitive to physical loading (i.e. gene-environment interaction) and thus modulate athlete health (and, by extension, enhance endurance performance). Specifically, if an athlete has a genetic predisposition towards low BMD or elevated risk of stress fracture, exercise training and/or diet could be modified to accommodate.

#### *1.2.4 Genetic associations with stress fracture*

There is a lack of conclusive evidence regarding external determinants of stress fractures (Wright et al., 2015) as mentioned in Section 1.1.5. In more recent times, the idea of a proposed genetic influence has been investigated primarily in military recruits, due to the abrupt increase in training, large training volumes and high prevalence of stress fractures (Lappe et al., 2008). Examples have included the calcitonin receptor (*CTR*) rs1801197 and *LRP5* rs2277268 polymorphisms, which were associated with femoral neck stress fractures in 72 Finnish military recruits (Korvala et al., 2010).

Participants who possessed the *CTR* C allele together with a *VDR* C-A haplotype were more protected from stress fractures, which may be due to the role of *CTR* in osteoclast mediated bone resorption (Pondel, 2000).

Furthermore, larger sized CAG androgen receptor (*AR*) gene repeats (>16) were more common in Israeli military personnel who had suffered stress fractures (23%) than those who had not suffered this injury (13%) (Yanovich et al., 2011). A higher number of CAG repeats within the *AR* gene are inversely associated with the transcriptional response to testosterone (Zitzmann et al., 2001) and deficiency in such hormones could influence bone metabolism and potential bone loss (Mohamad et al., 2016; Khosla, 2015).

Stress fracture susceptibility, in relation to genetics, has also been investigated in athletes for the first time recently, with findings suggesting that athletes with specific genetic variants may have an increased vulnerability to this injury (Varley et al., 2015; Varley et al., 2016; Varley et al., 2017). Interestingly, three of the same SNPs (*VDR* FokI rs2228570, *TNFSF11* rs1021188 and the loss of function *P2RX7* rs3751143) as mentioned above, alongside *TNFRSF11A* rs3018362, were associated with stress fracture incidence in the Stress Fracture in Elite Athlete (SFEA) cohort. However, a gain of function *P2RX7* SNP (rs1718119) was associated with reduced stress fracture occurrence. Functional expression of purinergic receptor P2X 7 primarily regulates configuration of osteoclasts (Agrawal et al., 2010), as well as augmenting bone formation via a cell-autonomous role that leads to stimulation of mineralisation (Panupinthu et al., 2008). This may explain why some *P2RX7* polymorphisms have also been associated with low baseline and accelerated bone loss in post-menopausal women (Gartland et al., 2012). *P2RX7* is a particularly interesting candidate gene in regards to potential gene-physical activity interactions and outcomes for BMD. Mice with a null mutation of *P2RX7* have been reported to show >73% reduced sensitivity

to mechanical loading (Li et al., 2005). Fluid shear stress increased Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) release in wild type osteoblast cells but had no effect was observed on PGE<sub>2</sub> release in knockout osteoblast cells. PGE<sub>2</sub> administration activates cortical bone modelling resulting in increased bone mass (Jee et al., 1990) and Li et al. (2005) suggested these findings indicate ATP signalling through *P2RX7* is important for mechanically induced release of prostaglandins by bone cells and subsequent bone formation. Consequently, variation in *P2RX7* SNPs could result in differing responses to mechanical loading and alterations to BMD, potentially influencing stress fracture susceptibility.

Although research investigating genetic influence on stress fracture has begun using the SFEA cohort, this was a loosely defined group, which comprised athletes of mixed abilities and from a range of sports. A more focussed approach, which removes the variability (i.e. loading/training patterns) introduced by incorporating athletes from different sports into one investigation, would be advantageous.

### **1.3 Future directions and conclusions**

There are numerous polymorphisms that need further exploration vis-à-vis BMD. In particular, gene-environment (i.e. gene-physical activity) interactions are likely to contribute substantially to inter-individual differences in BMD throughout the human lifespan. Exciting findings have been observed in regards to gene-physical activity interactions and genetic associations with stress fracture, particularly in variants of pathways involved in the adaptation of bone to mechanical loading, such as the RANK/RANKL/OPG system.

Therefore, the study of specific cohorts, who experience unusually high mechanical loads and who may display unusual bone phenotypes and/or possess genetic characteristics that differ from the norm, may provide novel insight into the area. Such



individuals include high-level athletes, who are at the extremes of human physiological capability, experience much greater environmental (mechanical) stress than most and might possess a genotype particularly suitable to tolerate those stresses.

GWAS or ideally whole genome sequencing (WGS) studies using athletic populations with their differentiating extreme phenotypes are, in principle, the next logical steps to identify key polymorphisms. Detailed study of gene function can follow. However, most GWAS designs cannot account for gene-gene/gene-environment interactions and only analyse SNPs with minor allele frequencies of more than 1%, not rare variants that may lie between 0.1-1% or even lower. Thus, GWAS is appropriate for the discovery of common variants that may confer low/moderate risk but are underpowered for the detection of rare variants, which may have a large influence on a complex phenotype according to the common disease/rare variant hypothesis (Li and Leal, 2008). Conducting GWAS or WGS studies is also extremely challenging due to the associated costs and difficulty in recruiting sufficiently large numbers of such a specific population. Even a panel of SNPs for investigation that is far lower in number than used in contemporary GWAS, for example 500 SNPs, would require a sample size of 1200 to detect an effect size of 0.02 in a continuous trait, assuming 80% statistical power, a minor allele frequency of 20% and an alpha level of 0.0001. Approximately the same size of sample would be needed for each group of a case-control study design, assuming the same parameters and an effect size (odds ratio) of up to 1.4 (Bouchard, 2011).

While the large cohorts necessary for GWAS and eventually WGS studies of BMD in athletes are built, smaller samples (steps towards building the bigger sample) can be used to test hypotheses about genetic variants emerging from GWAS in relevant clinical populations. Assessing bone and injury phenotype data in those athletes will also enhance understanding of any observed genotype-phenotype relationship (Wang

et al., 2013). A relatively homogenous group of athletes who experience high mechanical loads on some bone structures, such as endurance runners, would be suitable for this kind of investigation. Specifically, measuring areal BMD via DXA scanning, with a particular emphasis on the primary loading sites in this population, would probably provide appropriate data to combat some of the aforementioned challenges identified. It would be fascinating to discover whether those athletes have a genotype that enhances BMD, protects against the effects of the large volume of training required and reduces risk of stress fracture. One preliminary report (using just 14 participants) even documents an attempt to reduce the risk of tendon, ligament and bone injuries by modifying athlete training programmes based upon genetic characteristics (Goodlin et al., 2015). This illustrates the kinds of future applications possible in this field, *after* the more fundamental research has been conducted successfully.

#### **1.4 Identifying candidate genes**

Over 66 genetic loci have been associated with DXA-derived BMD/Osteoporosis via GWAS and almost 100 have been associated via candidate gene association study (Hsu and Kiel, 2012), with some of these variants having also been associated with stress fracture (Varley et al., 2015; Varley et al., 2016; Korvala et al., 2010). This vast number highlights the likely highly polygenic nature of BMD. Many of the genes discovered via GWAS have no known or limited understanding in regards to their potential biological function on bone. Only 7 of the >100 variants associated with BMD via candidate gene association study have also been associated at GWAS level (Hsu and Kiel, 2012). Many of these genes have not had further replication in different populations or cohorts via GWAS or in candidate gene association studies. Moreover, this means many candidate genes have not had replication in populations where environmental stimuli may be prominent, as would be the case of mechanical loading

in high-level weight-bearing athletes as highlighted previously in this chapter. Candidate gene-selection and the approach utilised to investigate potential associations with BMD is consequently, a difficult process.

GWAS allows identification of genomic loci in a large population of individuals by completing an expansive scan of the genome in those who may have a particular phenotype/condition of interest in comparison to those who do not have the phenotype by typically genotyping two hundred thousand up to two million variants (Estrada et al., 2012; Visscher et al., 2017). GWAS has been completed in individuals with osteoporosis, high bone mass (Gregson et al., 2018) and other bone-related conditions, such as Paget's disease (Albagha et al., 2010) as well as being implemented for tissue-specific pathway association analysis (Wang et al., 2017). Overall, although GWAS provides strong statistical power, they remain high in costs in comparison to candidate gene approach studies. Furthermore, the associated variants discovered by GWAS can be missed through excessive statistical power or the variants identified within adjacent loci can be correlated and in linkage disequilibrium (Wall and Pritchard, 2003). Loci in linkage disequilibrium can both exhibit significant genotype-phenotype association but only one may have a functional relevance for the phenotype in question. A candidate gene association study is different to GWAS in being hypothesis driven and aims to determine association between genetic variations and a particular phenotype. Candidate gene association studies can be utilised to further study specific variants that may have had strong associations at GWAS level (Teare, 2011). Replication of these specific SNPs, via a candidate gene approach may provide further evidence that the associations are not false-positive findings as discussed in Section 1.2. GWAS was not possible in this thesis and requires large sample sizes, which is unfeasible given that high-level endurance athletes who complete substantial volumes of mechanical loading are a sparse, homogenous group.

Consequently, utilising a candidate gene approach provides the most suitable method to investigate the highly evidenced BMD-associated genes, using the current clinical standard measurement of BMD (DXA) in a population that has had limited study on to date. Moreover, further understanding of the genes associated with BMD in athletic populations can add to the understanding of the genetics of athlete status and performance. Specifically, in relation to bone, whether certain runners are advantageously genetically predisposed to higher BMD and a subsequent reduction in stress fracture risk. A number of SNPs associated with health or fitness-related phenotypes have been explored in relation to athlete performance, injury and status (Guth and Roth, 2013) but investigation using this approach for bone is somewhat lacking. Therefore, approximately 20 SNPs were ranked on the basis of the volume and strength of evidence in relation to GWAS and/or case-control association and/or associated biological function with BMD. Based on available resources, the top 10 were then selected for investigation.

Consequently, ten candidate single nucleotide polymorphisms (SNPs) were identified for investigation within this thesis, from previous GWAS and/or candidate gene association studies, that each have a functional effect on bone physiology/metabolism (Table 1.3). Many of these chosen SNPs, for which specific overviews are detailed in the subsequent section of this review, have also shown potential gene-physical activity interactions or conflicting results in athletic populations. Further investigation of these SNPs in athletic cohorts will add to the current body of literature, extend our understanding of the genetic associations with BMD and provide new knowledge in regards to athlete status and performance.

**Table 1.3:** The candidate gene SNPs and their associated proteins selected for analysis for associations with status, performance, BMD and stress fracture incidence in high-level endurance athletes.

Candidate gene	Gene abbreviation and SNP	Candidate gene protein
axin1	<i>AXIN1</i> rs9921222	axin-1
BDNF antisense RNA	<i>BDNF-AS</i> rs6265	Non-protein coding
Collagen type I alpha 1 chain	<i>COL1A1</i> rs1800012	Collagen alpha-1(I) chain
Catechol-O-methyltransferase	<i>COMT</i> r4680	Catechol O-methyltransferase
LDL receptor related protein 5	<i>LRP5</i> rs3736228	Low-density lipoprotein receptor-related protein 5
Purinergic receptor P2X 7	<i>P2RX7</i> rs3751143	P2X purinoceptor 7
TNF receptor superfamily member 11a	<i>TNFRSF11A</i> rs3018362	Tumor necrosis factor receptor superfamily member 11A (RANK)
TNF receptor superfamily member 11b	<i>TNFRSF11B</i> rs4355801	Tumor necrosis factor receptor superfamily member 11B (OPG)
vitamin D receptor	<i>VDR</i> rs2228570	Vitamin D3 receptor 1,25-dihydroxyvitamin D3 receptor
Wnt family member 16	<i>WNT16</i> rs3801387	Wnt-16

#### 1.4.1 Candidate genes of interest

##### *AXIN1* rs9921222

*AXIN1* is a Wnt signalling loci, which is known to influence the mechanosensitivity of the skeleton (Robinson et al., 2006). *AXIN1* is suggested to encode regulators of the Wnt signalling pathway, specifically as an element of the beta-catenin destruction complex (Baron and Kneissel, 2013; Stykarsdottir et al., 2016), and therefore, may influence bone mass by stimulating differentiation and replication of osteoblasts to enhance bone formation. The *AXIN1* rs9921222 SNP has been found to have strong associations with BMD via GWAS meta-analysis (Estrada et al., 2012). Specifically,

the T allele of the *AXIN1* rs9921222 was associated with lower femoral neck and lumbar spine BMD in a large adult cohort (n > 50,000) but was not associated with fracture in 31,016 cases and 102,444 controls (Estrada et al., 2012). The rs9921222 SNP has also been reported to have nominal associations with BMD via gene-physical activity interactions in children and adolescents (Mitchell et al., 2016). Mechanical loading leads to an increase in Wnt production by osteocytes, which activates the signalling pathway (Klein-Nulend et al., 2012), however, the potential gene-physical activity interaction mechanism is unknown. Further functional study of *AXIN1* on bone is required to confirm potential influence on bone but *AXIN1* presents a noteworthy candidate to explore gene-physical activity interactions due to the results found from investigation so far. Although limited research has been conducted into the potential *AXIN1*-physical activity interactions on BMD, no studies have been conducted utilising athletic populations where potential influences on other BMD-related phenotypes such as stress fracture, can be explored additionally.

#### *BDNF-AS rs6265*

Brain-derived neurotrophic factor antisense RNA (*BDNF-AS*) encodes brain-derived neurotrophic factor and has been primarily associated with the repair and plasticity of neurons (Lipsky and Marini, 2007). One specific SNP, the C to T missense variation at nucleotide 196 resulting in a valine to methionine (Val66Met) substitution at codon 66 (Egan et al., 2003), has been associated with influencing such phenotypes. At the *BDNF* opposite strand, the *BDNF-AS* gene is transcribed in a reverse complimentary direction that produces alternative RNA transcripts whose fifth exon overlaps with the protein coding exon of *BDNF*, influencing *BDNF* expression. Inhibition of *BDNF-AS* expression can upregulate *BDNF* mRNA significantly, resulting in increased protein levels. Consequently, alteration of *BDNF-AS* expression through genetic variation could ultimately effect BDNF-associated phenotypes such as neuron plasticity. This

alteration could also influence bone phenotypes as proposed in recent mice studies that have demonstrated *BDNF* knockdown can inhibit osteoblast differentiation, resulting in increased bone formation (Guo et al., 2016). This strong influence on osteoblast differentiation via *BDNF* through genetic variation in *BDNF-AS* could have a substantial impact for BMD and potential influence on stress fracture via potential disturbances in bone remodelling. *BDNF* influence on BMD has also been observed in humans where AA genotype is associated with lower spine and hip BMD in Caucasians (Deng et al., 2013). Overall, limited research into *BDNF-AS* association with human phenotypes exists, particularly that which assesses a potential influence of *BDNF-AS* on BMD, and therefore, additional study is still warranted in both candidate-gene association and functional studies.

#### *COL1A1 rs1800012*

*COL1A1* encodes Collagen alpha-1(I) chain, which is the most abundant protein in bone and presents one of the most extensively studied genes in relation to human tissue. *COL1A1* SNPs have been associated with both soft-tissue injury and bone phenotypes (Posthumus et al., 2009; Ficek et al., 2013). One specific SNP in particular, rs1800012, has received extensive research, which has indicated that the TT (CC), rather than the CC (AA) genotype is associated with lower hip and lumbar spine BMD as well as increased risk of osteoporotic fracture in meta-analysis (Jin et al., 2011). Functional studies have also demonstrated that the rs1800012 polymorphism is associated with alterations in *COL1A1* transcription and protein production, influencing bone mass (Mann and Ralston, 2003). Specifically, possessing the T (C) allele of the *COL1A1* rs1800012 SNP increases the amount of transcript for  $\alpha 1$  chain, which has been suggested to result in the formation of collagen homotrimers associated with degenerative bone microarchitecture (Mann et al., 2001; Grant et al., 1996; Dytfeld et al., 2016). Additionally, *COL1A1* gene expression has been shown

to increase following mechanical loading in rats, thus emphasising the potential impact of mechanical loading that could be genotype-dependent for *COL1A1* variants (Mantila Roosa et al., 2011). Research exploring *COL1A1* genotype associations has been conducted into athletic populations but this has primarily focused on soft tissue injuries and not BMD or bone phenotypes (Posthumus et al., 2009). To the author's knowledge, there exists only one study thus far that has investigated a potential association of *COL1A1* rs1800012 with bone phenotypes in athletes, which observed no association with stress fractures (Varley et al., 2018). Consequently, additional study on BMD in this population is certainly worthwhile due to the functional influence of *COL1A1* rs1800012 in bone and previous findings on bone phenotypes in non-athletic populations. Athletic success is a complex phenotype influenced by a substantial number of factors but *COL1A1* rs1800012 provides an extremely interesting SNP to investigate potential associations with BMD and athlete status due to previous associations with soft-tissue injury in this population.

### *COMT* rs4680

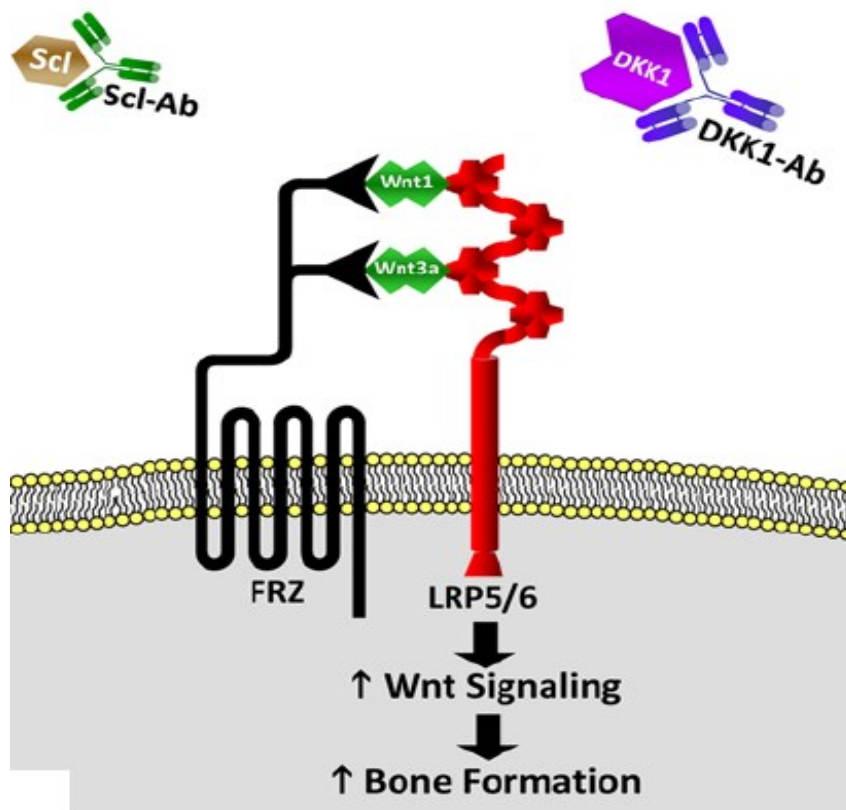
The *COMT* gene encodes catechol O-methyltransferase and is important for the metabolic degradation of dopamine. The rs4680 SNP has been associated with decision making (He et al., 2012), prefrontal cognition (Malhotra et al., 2002) and Alzheimer's disease (Serretti and Olgiati, 2012) but it has also provided interesting results in relation to BMD. COMT catalyses the methylation of catechol oestrogens to methoxy oestrogens (inactive metabolites) and thus, lower COMT enzyme activity results in greater 16-hydroxy-oestradiol, which retains oestrogenic activity and enhances BMD (Lorentzon et al., 2007). Consequently, both higher oestradiol serum concentration (Eriksson et al., 2004) and higher BMD (Eriksson et al., 2005) have been reported in lower enzyme activity AA genotypes. Conflicting results regarding which *COMT* rs4680 genotype is more advantageous for BMD, however, have been



observed (Gonçalves et al., 2015), which may be due to interactions of mechanical loading. The rs4680 SNP has been shown to influence the association between physical activity and BMD, suggesting that certain genotypes may be particularly important for BMD in individuals with low physical activity levels as mentioned previously in Section 1.2.2 (Lorentzon et al., 2007). A *COMT* genotype-interaction, thus, may be present and the potential regulation of the BMD response to mechanical loading may be due to the involvement of oestrogen receptors as facilitators in a number of key pathways by which mechanical strain stimulates bone formation (Galea et al., 2013). Investigation into *COMT* and BMD is limited and thus, further study is warranted to provide greater evidence of potential associations with BMD and/or a gene-physical activity interaction.

#### *LRP5 rs3736228*

Activation of Wnt/ $\beta$ -catenin (canonical) signaling increases the sensitivity of osteoblasts to mechanical loading, which can occur via Wnt binding to low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/LRP6) co-receptors (Robinson et al., 2006; Krishnan et al., 2006).



**Figure 1.11:** Wnt proteins (1 and 3a) bind with LRP5/LRP6 to form a complex with Frz receptors (frizzled receptor family) resulting in increased Wnt signaling and increased bone formation. Figure taken from (Rocheffort, 2014).

It has been proposed that the mediation of Wnt signaling via different *LRP5* variants can both enhance and decrease BMD (Ferrari et al., 2005). Loss-of-function mutations in *LRP5* are also responsible for low bone mass disorders, such as osteoporosis pseudoglioma, whereas gain-of-function mutations have been suggested to cause high bone mass syndromes (Levasseur et al., 2005). Furthermore, *LRP5* variants, such as C135242T, have been associated with BMD variability in the general population (Koay et al., 2004) and ds2306862 in osteoporotic individuals (Mizuguchi et al., 2004), highlighting the strong influence *LRP5* may have on bone metabolism. Particularly interesting findings have been reported for the rs3736228 SNP influence on BMD, which was dependent upon volume of physical activity completed (Kiel et al., 2007). Specifically, rs3736228 TT genotypes were associated with lower BMD in men with higher physical activity level but higher BMD in those with a lower physical activity

level. The authors hypothesised that the C to T allele substitution alters LRP5-mediated Wnt signalling in the case that the catabolic signals induced from the mechanical loading prevails over anabolic signalling (Kiel et al., 2007). This was also evidenced when expressing alleles in the context of their haplotype *in vitro*, where the T allele was associated with a decreased response to canonical Wnt3a signalling in comparison to the C allele. Overall, *LRP5* rs3736228 has reported strong associations with BMD and osteoporotic fracture via both GWAS and candidate gene association studies in young and older adults (Richards et al., 2008; Saarinen et al., 2007). Moreover, similarly to *AXIN1* rs9921222, *LRP5* rs3736228 has demonstrated initial associations with physical activity and exciting outcomes for BMD, but has yet to be investigated for associations with these phenotypes in athletic populations and therefore represents a candidate variant deserving further investigation in this population.

#### *P2RX7* rs3751143

Functional expression of purinergic receptor P2X 7 primarily regulates configuration of osteoclasts (Agrawal et al., 2010), as well as augmenting bone formation via a cell-autonomous role that leads to stimulation of mineralisation (Panupinthu et al., 2008) and thus may be important for increasing BMD. *P2RX7* SNPs have also been demonstrated to mediate interleukin-1 beta (IL1B) which has been shown to facilitate osteoclast regulation and decrease bone collagen and non-collagen protein synthesis (Nemetz et al., 2001). Similarly, *P2RX7* mediation on interleukin-18 (IL18) is also apparent, IL18 has been shown to increase OPG expression in osteoblastic cells and consequently inhibit bone resorption (Makiishi-Shimobayashi et al., 2001). A number of *P2RX7* polymorphisms have been explored in relation to BMD in osteoporotic populations as well as stress fracture incidence in elite athletes and military recruits (Varley et al., 2016). One particular *P2RX7* variant (rs3751143) has been shown to

have effects on receptor functioning, whilst mice with a null mutation of *P2RX7* have exhibited >73% reduced sensitivity to mechanical loading (Li et al., 2005). Possessing the rs3751143 null CC genotype results in a loss of function whereas heterozygotes have been proposed to have half the receptor functioning (Gu et al., 2001). This loss of function could explain why the C allele has been associated with lower hip and lumbar spine BMD in women (Wesselius et al., 2013). C allele associations with stress fracture have also been observed both in military recruits and elite athletes (Varley et al., 2016) from the elite stress fracture cohort (SFEA). The SFEA comprised a large number of athletes that were of mixed abilities across a range of sports and thus, other factors such as physical and training characteristics may influence stress fracture susceptibility as discussed previously in Chapter 1. *P2RX7* rs3751143 alongside a number of other candidate genes such as *TNFRSF11* rs1021188 and *SOST* rs1877632 have been associated with stress fracture in the SFEA cohort, which is the only study so far to investigate genetic associations with stress fracture incidence in athletes. Consequently, further research utilising homogenous cohorts representing one sport would provide further evidence on these specific variant associations with BMD and physical activity phenotypes. *P2RX7* rs3751143 therefore, remains a prime candidate for further study so that a greater understanding of the potential specific contribution *P2RX7* may have to BMD and other bone phenotypes such as fracture in diverse populations.

#### *TNFRSF11A* rs3018362

*TNFRSF11A* is located at 18q22.1 on the chromosome and encodes RANK which plays a fundamental role in osteoclast differentiation and function (Albagha et al., 2010). Binding of RANK to RANKL initiates signalling pathways, such as Mitogen activated protein kinase (MAPK), resulting in activation of osteoclasts (Wada et al., 2006). Genetic regulation of osteoclastogenesis via RANK has been supported in

functional studies. For example, nullizygous mice exhibit osteoporosis due to osteoclast absence and the associated defect in bone resorption and remodelling (Li et al., 2000). This potential strong genetic influence of *TNFRSF11A* on bone has been reported across a number of SNPS, with *TNFRSF11A* rs3018362 proposed as a prime candidate from previous study. The A allele has been associated with Paget's disease and reduced BMD via GWAS (Albagha et al., 2010). Conflicting results, however, have reported no *TNFRSF11A* rs3018362 genotype associations (reaching genome-wide significance) with hip BMD in 5861 Icelandic adults. The *TNFRSF11A* rs3018362 SNP has also been associated with stress fracture incidence in athletic populations as evidenced within the SFEA cohort (Varley et al., 2015). Overall, strong functional evidence has been reported in regards to RANK and initiation of BMD-relevant signalling pathways, although contradictory findings regarding the *TNFRSF11A* rs3018362 association with BMD and other bone phenotypes exist. The difference in findings could be due to the influence of other factors on RANK expression. For example, interleukin-1 (IL1) and IL6 are both mediators of RANK and RANKL expression and thus can effect bone resorption by facilitating osteoblast and osteoclast interaction (Steeve et al., 2004). Consequently, different determinants, other than *TNFRSF11A* genotype, will regulate RANK expression and ultimately impact BMD, as is likely to be the case for a number of other genetic variants involved within bone metabolism pathways. Further research is therefore required to provide more evidence to the specific contribution of *TNFRSF11A* variation on BMD across different populations. Due to the functional evidence supporting the impact of RANK in bone metabolism, it would be particularly interesting to explore if *TNFRSF11A* rs3018362 genotype-dependent differences in BMD are present in athletic populations who are completing large volumes of mechanical loading.

### *TNFRSF11B rs4355801*

The human TNF receptor superfamily member 11b (*TNFRSF11B*) gene encodes the protein OPG, which is secreted by osteoblasts and aids in regulating bone resorption by inhibiting differentiation and activation of osteoclasts. Consequently, alongside *TNFRSF11A* (RANK), OPG forms part of the RANK/RANKL/OPG pathway which regulates bone metabolism, through initiation of osteoblastic or osteoclastic activity (Tyrovola and Odont, 2015). OPG-deficient mice have been found to develop early onset osteoporosis, and increased tissue mRNA expression has been observed in participants who possess specific haplotypes, accompanied with reduced BMD, which may be due to the increased expression resulting in stimulated osteoclast activity (Takács et al., 2010). Alongside these findings, *TNFRSF11B* rs4355801 genotype appears to influence BMD in humans in association studies. Specifically, the *TNFRSF11B* rs4355801 A allele has been associated with lower BMD via GWAS in adults (Richards et al., 2008) as well as cortical BMD in adolescents (Paternoster et al., 2010). Alongside the association with BMD in GWAS, the “risk” A allele (which explained 0.4% of the variance at both the lumbar spine and femoral neck) was also associated with reduced *TNFRSF11B* expression. Although these findings suggest the A allele can negatively impact BMD, the proposed “advantageous” G allele has been associated with stress fracture in the SFEA cohort (Varley et al., 2015). In the runners specifically, those who possessed at least one copy of the G allele had a greater risk of stress fracture. Consequently, these findings contradict the majority of other studies exploring *TNFRSF11B* rs4355801 and thus, further study is required to gather a greater understanding of which allele may be the more “advantageous” for BMD and if this may vary depending upon environmental stimuli such as mechanical loading.

## *VDR rs2228570*

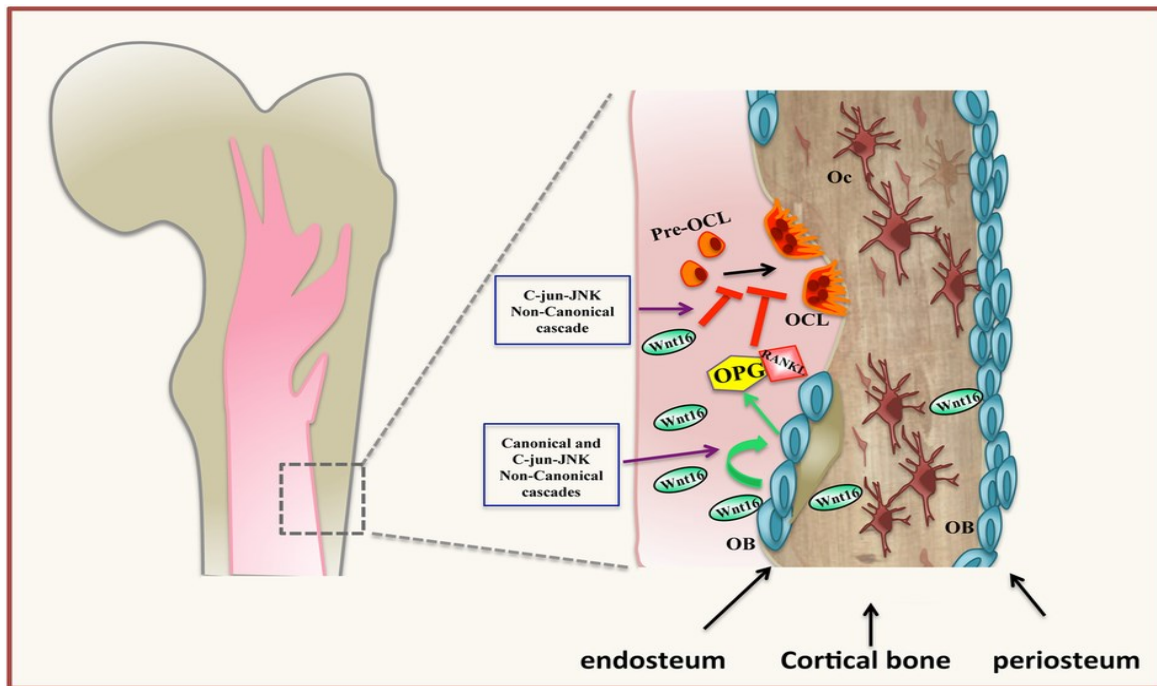
Vitamin D receptor (*VDR*) controls the transcription of other genes such as osteocalcin that are instrumental for calcium absorption and bone formation (Moran et al., 2014). Specifically, vitamin D functions as the ligand for *VDR*, which facilitates calcium and phosphate absorption to induce bone mineralisation (Haussler et al., 1998). A direct effect of osteoblastic/osteocytic *VDR* signalling on bone remodelling has also been proposed, although specific understanding of this notion is still lacking and largely depends on calcium balance (Lieben and Carmeliet, 2013). The potential association of *VDR* with BMD and/or fracture has also been supported across a number of different SNPs (Bsm1 rs1544410, Apa1 rs7975232 and Taq1 rs731236) in various cohorts, such as pre and postmenopausal women (Riggs et al., 1995; Horst-Sikorska et al., 2007; Ji et al., 2010; Marozik et al., 2013). One particular SNP (FokI rs2228570) has been extensively studied across a number of populations with contradictory findings have been observed (Gentil et al., 2009). This particular polymorphism, FokI (rs2228570), exhibits a C to T transition that creates an upstream initiation codon, leading to the production of *VDR* proteins that are three more amino acids in length. Consequently, this transition influences *VDR* protein expression and may explain why higher BMD has been reported in those with CC genotype (Arai et al., 1997; Colin et al., 2000; Uitterlinden et al., 2004; Ames et al., 1999) as described in Section 1.2.3. *VDR* rs2228570 has also been explored in relation to gene-physical activity interactions, where the ff genotype was associated with higher BMD (rather than the FF) genotype in postmenopausal women (Gentil et al., 2009). However, in athletic populations, opposing results have been reported. Significantly higher total BMD in 84 weight-bearing athletes than 80 controls was observed in the FF (7.7%) and Ff (6.9%) but not ff (1.8%) genotypes for the *VDR* FokI rs2228570 polymorphism, whilst significantly lower total BMD was only observed in the FF (-4.5%) genotype when comparing

swimmers with a control group (Nakamura et al., 2002a). Overall, support for both alleles with a physical activity interaction exists in the literature and therefore, the specific population investigated in regards to age or sex and the type of physical activity completed may be accounting for the differences in the findings (Gentil et al., 2009; Rabon-Stith et al., 2005). *VDR* rs2228570 is one of the more extensively studied SNPs in relation to BMD, particularly in regard to physical activity. Further study, however, is required to determine which allele or genotype may be more beneficial for BMD and if this can be explained by differences in genotype sensitivity to the mechanical loading completed by athletic populations.

#### *WNT16* rs3801387

A number of Wnt ligands exist with some activating through the canonical pathway and others through the non-canonical pathway (Garcia-Ibarbia et al., 2013). Wnt16 is proposed to signal via the non-canonical pathway (Clements et al., 2011) but has also been proposed to signal via the canonical pathway and is a key regulator of osteoblast-to-osteoclast communication and consequently influence bone mass (Figure 1.12) (Gori et al., 2015). Expression of canonical Wnt target genes has also been observed to be substantially lower in *WNT16* knockout compared to wild type mice (Movérare-Skrtic et al., 2014).





**Figure 1.12:** Wnt16 is expressed in osteoblasts on the outer surface of cortical bone. Wnt16 signals via the canonical Wnt pathway to regulate OPG expression, which results in OPG functioning as a decoy receptor for osteoblast and osteoclast RANKL expression. The osteoblast-expressed Wnt16 can also impact osteoclasts precursors to regulate osteoclastogenesis via the non-canonical pathway. Figure taken from (Gori et al., 2015).

One particular *WNT16* SNP (rs3801387) has been reported to influence BMD in a number of investigations. The A allele of the rs3801387 was one of the more strongly associated SNPs with lower lumbar spine and femoral neck BMD as well as osteoporotic fracture in the Estrada et al. (2012) meta-analysis. An A allele association with lower LBMD has also been observed in candidate gene association studies (Hendrickx et al., 2014), accompanied with reduced *WNT16* expression and accounting for up to 1.8% of variance in total-body BMD in humans (Medina-Gomez et al., 2012). *WNT16* remains particularly interesting as age-specific effects of *WNT16* association on BMD have been reported which could suggest specific genetic determination of bone accrual in children and a protective effect against osteoporosis in later life. Additionally, *WNT16* rs3801387 (similarly to *AXIN1* rs9921222) has been reported to interact with physical activity and demonstrated nominal associations with

BMD (Mitchell et al., 2016). Despite the considerable number of studies suggesting associations with BMD and evidence from functional investigation, *WNT16* rs3801387 was not associated with stress fracture in the elite athletes of the SFEA cohort (Varley et al., 2017). Overall, due to the prior literature proposing *WNT16* rs3801387 association with BMD but not stress fracture occurrence in elite athletes, further study is needed in athletic populations to elucidate the exact nature of *WNT16* rs3801387 contribution to BMD and potential associations with physical activity.

### **1.5 Aims and objectives**

Consequently, the overall aim of the current thesis was to investigate the genetic associations with BMD, stress fracture incidence and performance in high-level endurance runners and compare these to a non-athlete cohort to explore genotype-physical activity interactions. More specifically, the objectives were:

- 1) To compare BMD between high-level endurance runners and non-athlete controls and identify genetic associations of 10 SNPs (*AXIN1* rs9921222, *BDNF-AS* rs6265, *COL1A1* rs1800012, *COMT* rs4680, *LRP5* rs3736228, *P2RX7* rs3751143, *TNFRSF11A* rs3018362, *TNFRSF11B* rs4355801, *VDR* rs2228570, *WNT16* rs3801387), individually and collectively, with BMD in these two populations.
- 2) To compare BMD in high-level endurance runners who had suffered a stress fracture in comparison to those who had not and to determine whether the 10 aforementioned SNPs, individually and collectively, were associated with stress fracture incidence in high-level endurance runners.
- 3) To determine whether the 10 aforementioned SNPs were associated with marathon personal best time in high-level endurance runners.

- 4) To determine any cohort-dependent differences in BMD-genotype associations for the 10 aforementioned SNPs.

# **Chapter 2:**

## **General methodology**

## **2.0 General Methodology**

The full method is listed within this chapter from which subsequent chapters use some or all of the participants and/or experimental procedures.

### ***2.1 Participants and participant recruitment***

All experimental procedures were conducted in accordance with the guidelines in the Declaration of Helsinki (World Medical Association, 2013) and approved by the local Ethics Committee of Manchester Metropolitan University. The total participant population comprised 581 Caucasian middle to long distance (endurance) runners (345 males, 236 females) who completed in events ranging from 3000 m to marathon and 559 healthy non-athlete controls. A self-report questionnaire (Appendix 1) detailing performance history, injury history, sporting activity in childhood and menstruation status/history for females was completed by the runners. Non-athletes completed a questionnaire designed to assess general health and physical activity levels (Appendix 2). Runners were primarily recruited from the London Marathon Expo 2012–2015 as well as national/regional athletic clubs and organisations, whilst the non-athlete control group were recruited through mail-outs, posters and word of mouth. Personal best (PB) race time was verified by official race chip timings through individual race result websites, the power of 10 (<http://www.thepowerof10.info/>) and/or the International Association of Athletics Federations (IAAF) (<https://www.iaaf.org/home>). Runners were included if they had completed at least one official long distance event  $\geq 3000$  m in a time faster than a predetermined threshold (Table 2.1). The predetermined threshold time for each distance was chosen to ensure all athletes placed in at least the top 600 in the UK rankings for a calendar year based on the years 2012-2017. Average weekly running distance ranged from 15–110 miles and training hours per week ranged from 8-18 hours.

**Table 2.1:** PB selection criteria for both male and female runners.

<b>Distances</b>	<b>Males</b>	<b>Females</b>
3000 m	< 8 min 45 s	< 10 min 15 s
5000 m/5 km road	< 15 min 45 s	< 18 min 45 s
10000 m/10 km road	< 32 min 45 s	< 38 min 45 s
Half marathon	< 74 min 00 s	< 88 min 00 s
Marathon	< 2 h 45 min 00 s	< 3 h 15 min 00 s

## **2.2 Genetic analysis**

### *2.2.1. DNA sample collection*

Deoxyribonucleic acid (DNA) for each participant was obtained via one of the three methods below:

A 5 mL blood sample was collected by a trained phlebotomist from a superficial forearm vein into EDTA collection tubes and inverted 10–15 times, before being aliquoted into 1.5 mL microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) and stored at -20°C. Approximately 75% of participant DNA was obtained from whole blood samples, whilst the remaining 25% was obtained via saliva sample (23%) or buccal swabs (2%). Saliva samples were collected following a minimum 30-minute abstinence from food and drink into Oragene DNA OG-500 collection tubes (DNA Genotek Inc., Ontario, Canada) in accordance with the manufacturer's guidelines before being stored at room temperature. Although blood sampling is cheaper and potentially quicker for collecting large sample sizes of genomic data, saliva or buccal swabs are less invasive and were the preferred method for some participants (Feigelson et al., 2001). Buccal cell samples were also collected after a minimum 30-minute abstinence from food and drink. Participants brushed one OmniSwab collection tip (Whatman Sterile Omniswab, GE Healthcare, USA) against the inside of one cheek for 30 s before

repeating this with a second swab on the opposite cheek to obtain two samples from each participant. Each collection tip was ejected into a 2 mL microcentrifuge tube and stored at -20°C. All samples were stored anonymously through coding and labelling in accordance with the Human Tissue Act (2004) and only members of the research team had access to this information and data.

### *2.2.2 DNA extraction*

DNA was extracted using the Qiagen QIAcube spin protocol (Qiagen, Crawley, UK) and the buffers contained in the Qiagen DNA Blood Mini Kit (Qiagen) for whole blood, saliva and buccal samples in accordance with the manufacturer's guidelines. DNA isolation from 200 µL whole blood or saliva required cell lysing with protease and AL buffer during incubation at 56°C for 10 min. Following centrifugation and the addition of ethanol, the resultant lysate was centrifuged at 8000 rpm for 60 s to allow silica gel membrane binding to occur. Removal of proteins, nucleases and other impurities was achieved following additional buffer-centrifugation cycles before elution of the remaining solution with 200 µL of AE buffer into a 1.5 mL microcentrifuge tube. Buccal swab extraction followed the same protocol as above for whole blood, however, an additional stage of transferring the lysate into sterile 2 mL microcentrifuge tubes prior to the DNA purification phase was required. The automated QIAcube was used to standardise these procedures and could complete this process on a maximum of 12 samples at a time.

### *2.2.3 DNA genotyping*

All participants were genotyped for the 10 SNPs on two different machines using the fluorophore-based detection technique of TaqMan real-time polymerase chain reaction (PCR), which requires amplification of a section of DNA overlapping the specific SNP being genotyped. To achieve amplification, forward primers were used to identify the

starting point of the DNA segment and reverse primers used to identify the end-point. Allele-specific probes, identified by either VIC or FAM (Table 2.2) attached to their respective complementary sequences, emitted a different fluorescent dye that could be distinguished by the respective PCR machine used. End-point fluorescence measurement of VIC and FAM determined the different genotypes (Figures 2.1 and 2.2) and results were analysed using the software supplied by the respective manufacturers of each PCR machine.



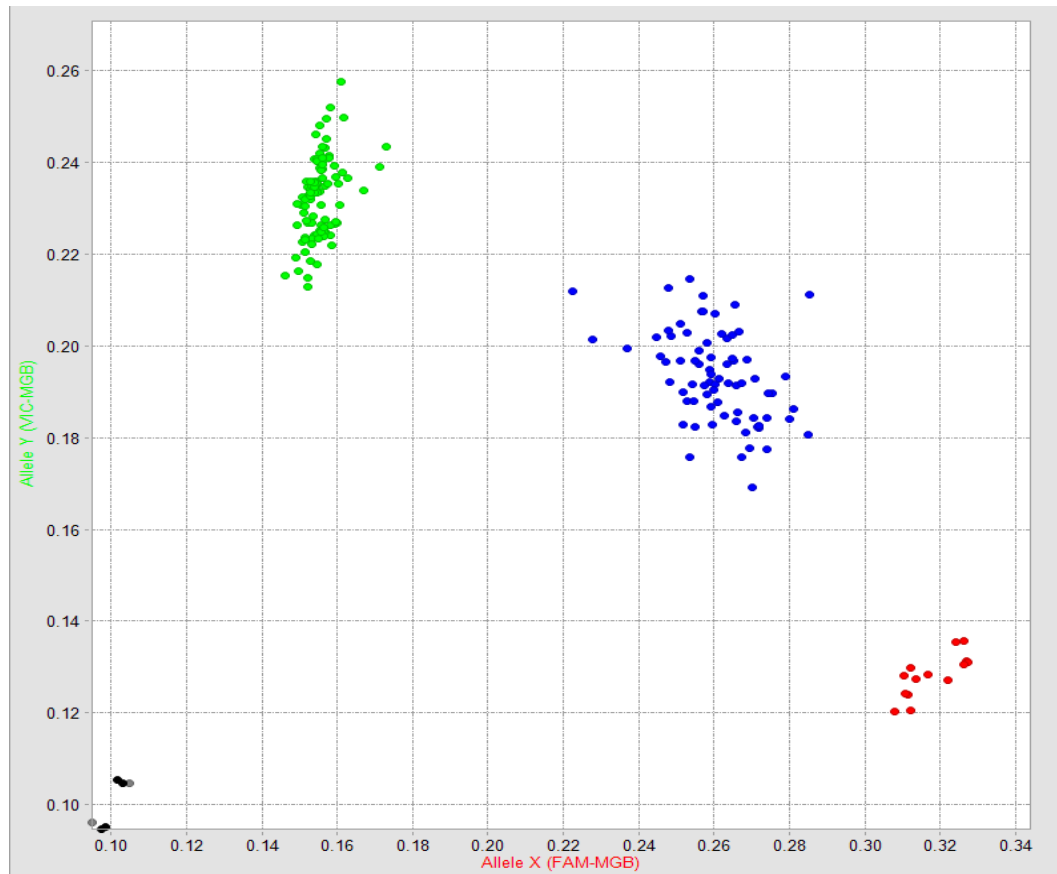
**Table 2.2:** SNPs used in genotyping and identification of allele-specific probes.

<b>SNP</b>	<b>VIC</b>	<b>FAM</b>	<b>Context Sequence(VIC/FAM)</b>
<i>AXIN1</i> rs9921222	C-allele	T-allele	GCTCTGTGTTAGCTCCATCTTCTCT[C/T] ATGACGGGGCCTTCGGAAACACCAA
<i>BDNF-AS</i> rs6265	C-allele	T-allele	TCCTCATCCAACAGCTCTTCTATCA[C/T] GTGTTCGAAAGTGTCAGCCAATGAT
<i>COL1A1</i> rs1800012	A-allele	C-allele	GGGAGGTCCAGCCCTCATCCCGCCC[A/C] CATTCCCTGGGCAGGTGGGGTGGCG
<i>COMT</i> rs4680	A-allele	G-allele	CCAGCGGATGGTGGATTTGCTGGC[A/G] TGAAGGACAAGGTGTGCATGCCTGA
<i>LRP5</i> rs3736228	C-allele	T-allele	GACTGTCAGGACCGCTCAGACGAGG[C/T] GGACTGTGACGGTGAGGCCCTCCCC
<i>P2RX7</i> rs3751143	A-allele	C-allele	CCTGAGAGCCACAGGTGCCTGGAGG[A/C] GCTGTGCTGCCGAAAAAGCCGGGG
<i>TNFRSF11A</i> rs3018362	A-allele	G-allele	ATCATCTTACCTACACCAGGTTTAC[A/G] TTTTCCATCTTAGAGTTATACAGGA
<i>TNFRSF11B</i> rs4355801	A-allele	G-allele	TAAACAGGTGTACAGGTCTCAATAA[A/G] TGGGTGGTAGGTGTCAGGGAAAGTC
<i>VDR</i> rs2228570	A-allele	G-allele	GGAAGTGCTGGCCGCCATTGCCTCC[A/G] TCCCTGTAAGAACAGCAAGCAGGCC
<i>WNT16</i> rs3801387	A-allele	G-allele	TGATTCCCACTTGGTTTCTGACACC[A/G] TTCCTAAACTTATGACTCCAAGAAT

Genotyping and subsequent analysis was completed in duplicate using either the (1) Fluidigm EP1 (Fluidigm, Cambridge, UK) or the (2) StepOnePlus (Applied Biosystems, Paisley, UK) as detailed below:

### (1) Fluidigm EP1

The majority of samples (95%) were genotyped for the 10 SNPs by combining 2 µL GTXpress Master Mix (X2) (Applied Biosystems), 0.2 µL 20X Fast GT Sample Loading Reagent (Fluidigm), 0.2 µL nuclease-free H<sub>2</sub>O and 1.6 µL of purified DNA into each well of a 192x24 microchip plate. Negative controls were also placed into 4 wells on each 192x24 microchip, in which nuclease-free H<sub>2</sub>O replaced the DNA sample. Furthermore, 1.78 µL assay (20X) (Applied Biosystems), 1.78 µL 2X Assay Loading Reagent (Fluidigm) and 0.18 µL ROX reference dye (Invitrogen, Paisley, UK) were combined per assay inlet. An integrated fluid circuit controller RX (Fluidigm) was used to mix samples and assays using a Load Mix (166x) script. PCR was performed using a real-time FC1 Cycler (Fluidigm) GT 192X24 Fast v1 protocol. Denaturation began at 95°C for 120 s followed by 45 cycles of incubation at 95°C for 2 s and then annealing and extension at 60°C for 20 s. The 192X24 microchip plate was then placed into the EP1 Reader for end-point analysis. Genotyping analysis was performed with the Fluidigm SNP genotyping analysis software. Duplicates of all samples were in 100% agreement.

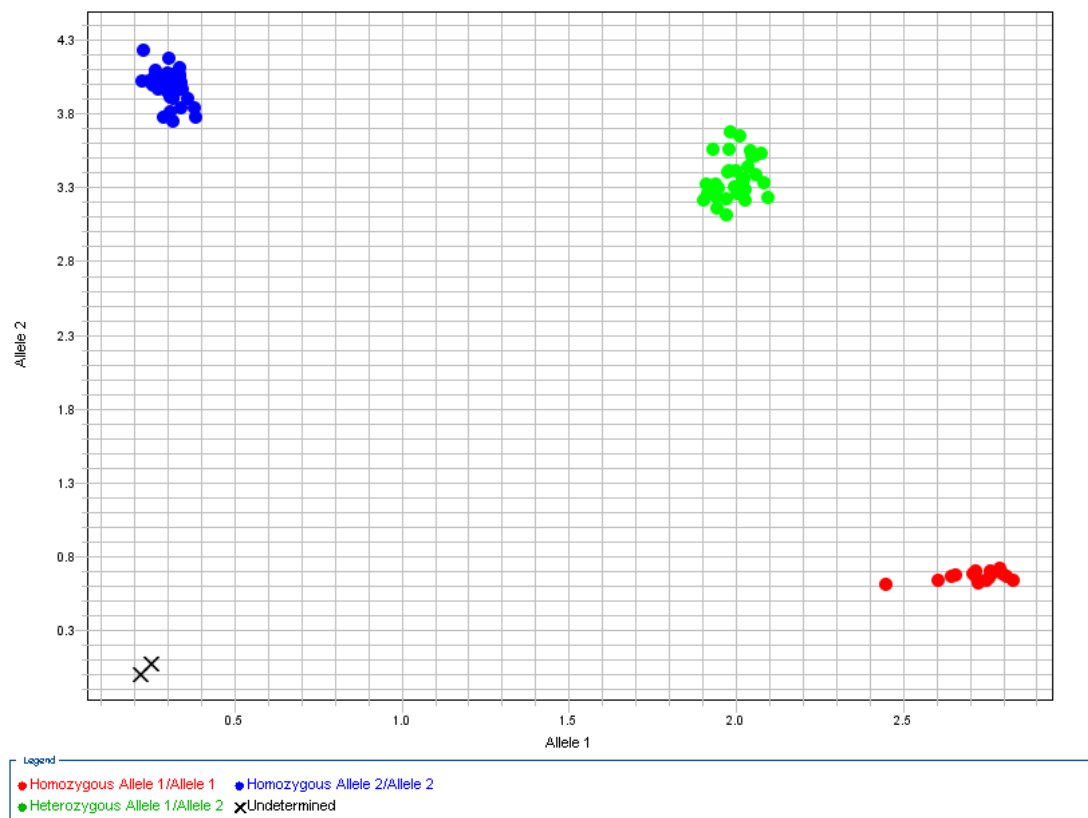


**Figure 2.1:** Example allelic discrimination plot of a single SNP using the Fluidigm EP1 system.

## (2) StepOnePlus

The remaining 5% of the samples were genotyped by combining 5  $\mu$ L Genotyping Master Mix or GTXpress Master Mix (Applied Biosystems), 4.3  $\mu$ L H<sub>2</sub>O, 0.5  $\mu$ L assay (Applied Biosystems), and 0.2  $\mu$ L of purified DNA (~9 ng), for samples derived from blood and saliva into wells on a 96-well plate (MicroAmp EnduraPlate Optical 96-Well Clear Reaction Plate, Applied Biosystems). For DNA taken from buccal swabs, 5  $\mu$ L Genotyping Master Mix was combined with 3.5  $\mu$ L H<sub>2</sub>O, 0.5  $\mu$ L assay mix, and 1  $\mu$ L DNA solution (~9 ng DNA). Negative controls were also placed into 2 wells on each 96-well plate, in which nuclease-free H<sub>2</sub>O replaced the DNA sample. Each well on a 96-well plate, therefore, contained a total reaction volume of ~10  $\mu$ L before being covered with an optical seal (MicroAmp Optical Adhesive Film, Applied Biosystems). PCR was performed using a StepOnePlus Real-Time PCR system (Applied

Biosystems). Denaturation began at 95°C for 10 min, with 40 cycles of incubation at 92°C for 15 s and then annealing and extension at 60°C for 1 min. Genotyping analysis was performed with StepOnePlus software version 2.3. Again, duplicates of all samples were in 100% agreement.



**Figure 2.2:** Example allelic discrimination plot of a single SNP using the StepOnePlus Real-Time PCR system.

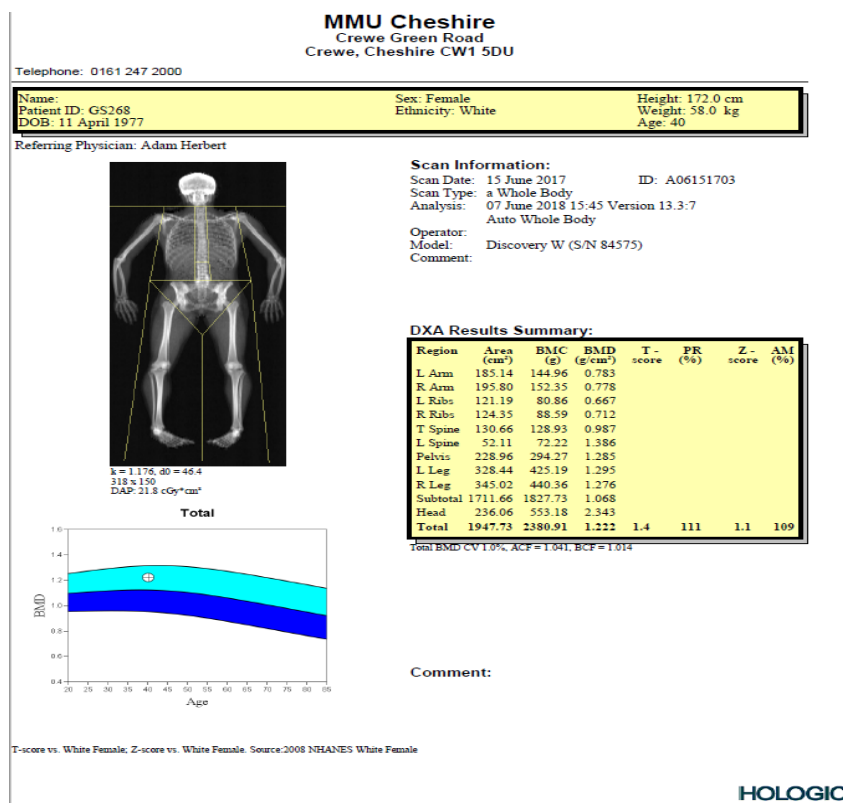
Reproducibility between the two PCR systems was evident when 94 samples for the *LRP5* rs3736228 variant were genotyped on both the StepOnePlus and Fluidigm PCR systems and were in 100% agreement.

## **2.3 Measurement of bone mineral density**

Bone mineral density (BMD) was measured using a Dual-energy X-ray absorptiometry (DXA) scanner (Hologic Discovery W, Vertec Scientific Ltd, UK) by one trained operator, consistent with the manufacturing guidelines. All participants wore a cotton examination gown, had no medically inserted metal implants and were instructed to remove any metal items prior to the scan. The participants lay supine in the centre of the machine table with arms by their sides (in a prone position) and legs internally rotated. To aid with internal rotation and comfort in this position, medical tape (Transpore™ Medical Tape, 3M™, USA) was wrapped around the outside of the feet (Figure 2.3). This participant placement followed manufacturing guidelines as well as ensuring the whole body was within the scanning boundaries and that enough space was left between arms, torso and legs to maximise accuracy of subsequent segmental analysis. The default whole-body scan mode was selected which emits dual energy (140/100 kVp) fan-beams to estimate body composition. The scanning region was 195 cm x 65 cm x 1.3 cm line spacing and 0.2 cm point resolution. Scan duration was approximately 7 minutes and the effective radiation dose to each participant was 8.4  $\mu$ sv (Blake et al., 2006). Subsequent segmental analysis for all scans were completed by the same trained operator using Physician's Viewer v6.1 software to obtain leg BMD as highlighted below (Figure 2.3). Specifically, leg BMD was obtained by calculating the average of the left and right leg BMD that was derived from the segmental analysis.



**Figure 2.3:** Participant positioning for whole-body DXA scanning.



**Figure 2.4:** Example DXA scan whole-body output following segmental analysis using Physician's Viewer v6.1 software.

## 2.4 Statistical analysis

Microsoft Excel version 2013 and SPSS for Windows version 23 (SPSS, Chicago, IL) were used for data analysis and specific statistical analysis for each experimental chapter is provided within the respective methods sections.

In brief, Pearson's Chi-square tests ( $\chi^2$ ) were utilised to test for Hardy-Weinberg equilibrium (HWE) and compare genotype and allele frequencies between runners, runner sub-groups and non-athletes for all 10 SNPs. One-way analysis of variance (ANOVA) was used to determine if runner PB differed between genotype groups for all 10 SNPs, individually and collectively as a total genotype score, whilst linear regression was also completed to identify if a total genotype score (TGS) could predict PB. Independent t-test analyses were completed to investigate any differences between the TGS of runners, runner sub-groups and non-athletes whilst receiver operator curve (ROC) area under the curve (AUC) analyses were conducted to determine if TGS was able to classify runners from non-athletes. Multiple analysis of variance (MANOVA) was used to compare BMD between the runners and non-athletes as well as compare BMD of the runners who had ever suffered a stress fracture with those who had no stress fracture injury history. Any genetic association with BMD was assessed via MANOVA and any interaction between the 10 investigated SNP genotypes and BMD in runners and non-athletes was also investigated via MANOVA analysis. BMD of the runners who had ever suffered a stress fracture compared to those with no stress fracture injury history was analysed via MANOVA before  $\chi^2$  tests were utilised to compare the genotype and allele frequencies of these two groups.

Alpha was set at 0.05 and Bonferroni or Benjamini-Hochberg corrections were implemented where appropriate (number of corrections were specific to and detailed

within each individual experimental chapter) to control false discovery rate.



# **Chapter 3:**

## **Bone mineral density in high-level endurance runners and non-athletes**

### 3.1 Introduction

Bone mineral density (BMD) is defined as the ratio of mass to the area or volume of bone (Ott et al., 1997) and is considered to be the primary predictor of osteoporotic fracture (Cranney et al., 2007). BMD is also important in the elite sporting environment, where athletes possessing lower BMD are found to be more at risk for stress fracture incidence (Myburgh et al., 1990; Bennell et al., 1999). Several factors such as diet, hormones, genetics and physical activity are known to influence BMD, contributing to the large variability that exists within the phenotype (Pluijm et al., 2001; Krall and Dawson-Hughes, 1993). Following physical activity, osteocytes detect shape and volume changes to increase or decrease the liberation of bone mediators, which consequently influences bone formation and resorption (Nakashima et al., 2011; Nakashima et al., 2012). This response to physical activity occurs across the lifespan, with children, adults and older adults, who complete a large volume of physical activity/exercise possessing greater BMD than those completing less physical activity/exercise (Chilibeck et al., 1995; Slemenda et al., 1991; Beck and Snow, 2003; Warburton et al., 2006). Athletic populations who complete large volumes of exercise, therefore, tend to possess higher BMD and bone mass than non-athletic individuals via the loading adaptation mechanisms mentioned above (Chilibeck et al., 1995). The loading characteristics of different sports, however, vary and so the BMD of athletes partaking different sports or disciplines also varies, particularly between different anatomical sites (Mudd et al., 2007; Bennell et al., 1997). Undertaking sports or exercise which express higher impacts through increased strain rates and higher peak-force loading, such as running, results in enhanced total or site-specific BMD (Fehling et al., 1995).

The influence of mechanical loading on BMD becomes a little more complex, however, in endurance athletes, such as long-distance runners. Higher leg BMD ( $0.14 \text{ g/cm}^2$ )

has been reported in male runners in comparison with non-athletes (Stewart and Hannan, 2000) and this enhanced BMD in running populations appears to continue into later life, with master athletes over 65 years old possessing higher BMD than non-active counterparts (Velez et al., 2008). Similarly, measurement of other bone parameters, such as cortical thickness at the primary loading sites (e.g. tibia), have also demonstrated higher bone mass in runners compared to non-active controls (Feldman et al., 2012). Investigation of total-body BMD at other anatomical sites that are not directly loaded, however, has produced differing results. Although higher total-body (8.6%), lumbar spine (12.2%), femoral neck (9.7%) and leg (13.2%) BMD has been found in female adolescent runners in comparison to controls (Duncan et al., 2002), most studies have reported low BMD in endurance runners at the non-loading sites. Hind et al. (2006) observed low lumbar spine BMD ( $<-1.0$  T-score) in 37% of the male runners aged 19-50 years investigated in comparison with a reference control database, whilst lower and low lumbar spine BMD (Z-score of  $-1.0$  to  $-2.0$ ) has also been reported in adolescent and adult female runners compared to control populations and reference databases (Pollock et al., 2010; Barrack et al., 2008b). Lower BMD was particularly evident in those exhibiting menstrual irregularities or dietary restraint (Barrack et al., 2008b; Barrack et al., 2008a). Low or lower BMD, therefore, can be observed in endurance runners at any site but particularly at non-loading sites, in those who may possess low energy availability and the subsequent suppression of bone formation. The influence of relative energy deficiency in sport (RED-S) and/or menstruation dysfunction on BMD may explain why runners, particularly females, can possess low or lower BMD than other runners or non-athletes (Schofield and Hecht, 2012; Pollock et al., 2010).

Overall, the majority of studies have found higher site-specific BMD but similar or lower total and non-loading site BMD (e.g. lumbar spine) in runners in comparison to non-

athletes due to the associated mechanical loading on the lower extremity (Schofield and Hecht, 2012). Further investigation is needed to understand the influence of mechanical loading on BMD in endurance runners in greater detail as well as to uncover the potential prevalence of low BMD and the impact this may have for health and performance in this population. Utilising large sample sizes of high competitive level (i.e. national and international) endurance runners is fundamental to extending our understanding of the importance of BMD in this population to ensure the development and implementation of effective injury prevention or management strategies to ultimately enhance performance. Although a reasonable number of studies have investigated BMD in endurance runners, it is difficult to offer direct comparisons or draw conclusions between these studies due to the substantial differences in methodological design. For example, many studies report on varied sample sizes comprising athletes of differing ability/standard, all of which are likely to increase the inter-individual variability within the phenotype. Most studies to date have been conducted on non-elite athletes (i.e. defined here as those who have not competed at international or national level), with only one study thus far comprising wholly of UK elite endurance runners (Pollock et al., 2010). Additionally, some studies did not obtain current/previous training hours and running distance to estimate bone loading or indicate high site-specific bone loading volume as expected of high-level endurance runners. If training volume/load is not accounted for, some studies may consist of runners who have completed substantially more training, and consequently applied more loading on the bone than another investigated cohort, which, along with variance in energy availability, may aid in explaining the conflicting results. Similarly, whilst several investigations do appear to use athletes of a similar ability/standard by recruiting only 'national' or 'regional' level athletes, the definitions of what constitutes national or regional level athletes are not always clear and consistent, again making comparisons between studies difficult (Swann et al., 2015). BMD comparison,

therefore, between these populations is difficult due to a probable difference in training load characteristics. Summarising the effect of loading on BMD and investigating BMD in elite endurance runners in comparison with non-athletes or other sports remains difficult due to limited investigation and the aforementioned variances in study design or population. In sports where ability/success is based on completion time for a specific distance, such as endurance running, criteria based on PB, rather than representative level for example, would allow for better assessment of athlete calibre and subsequent comparison of BMD between studies. Utilising a large homogenous cohort of high-level endurance runners of a similar competitive standard (based on PB), who are also likely to be completing similar training volumes and/or intensities, may somewhat alleviate the aforementioned issues when investigating BMD in endurance runners arising from using loosely defined populations and their potential confounding variables.

The primary aim of this chapter, therefore, was to investigate total body (TBMD), leg BMD (LBMD), lumbar spine BMD (LSBMD), total-body T-score and total-body Z-score in high-level endurance runners, at UK national standard for their respective PBs, in comparison to a non-athlete control group.

### **3.2 Method**

The investigated participants and protocols used in Chapter 3 have already been described in detail in Chapter 2, thus, only a brief description of these methods is detailed below.

#### *Participant characteristics*

Participants consisted of 103 high-level Caucasian runners (45 males, 58 females) and 112 ethnically matched non-athletes (52 males, 60 females) from the cohort as described in Table 2.1 of Chapter 2.

## *Protocol*

All runners completed a questionnaire detailing ethnic ancestry, as well as performance, injury and sporting history (Appendix 1). Female runners also completed a questionnaire detailing menstruation history that allowed potential identification of those who demonstrate, or have demonstrated, amenorrheic characteristics. Any runner who reported an absence of menses until after age 16 years or had undertaken 6 months without menstruation were identified as potentially amenorrheic (Gordon and Nelson, 2003). This questionnaire was utilised to investigate any potential effect of amenorrhea on BMD in the female runners. Non-athletes completed a questionnaire detailing ethnicity, general health and physical activity level to establish matched ethnic ancestry and ensure no history of high-level sporting competition (Appendix 2). All participants completed a whole-body DXA scan to gather BMD ( $\text{g}/\text{cm}^2$ ) data with whole-body and segmental analysis utilised to obtain total-body BMD (TBMD), leg BMD (LBMD) and lumbar spine BMD (LSBMD). Total-body T-score (the number of standard deviations above or below the mean for a sex- and ethnically-matched healthy 30 year-old adult in comparison to the DXA machine manufacturer's reference database, National Health and Nutrition Examination Survey, 2008) and total-body Z-score (the number of standard deviations above or below the mean for the patient's age, sex and ethnicity in comparison to the same database) were also acquired via the DXA scan and subsequent analysis.

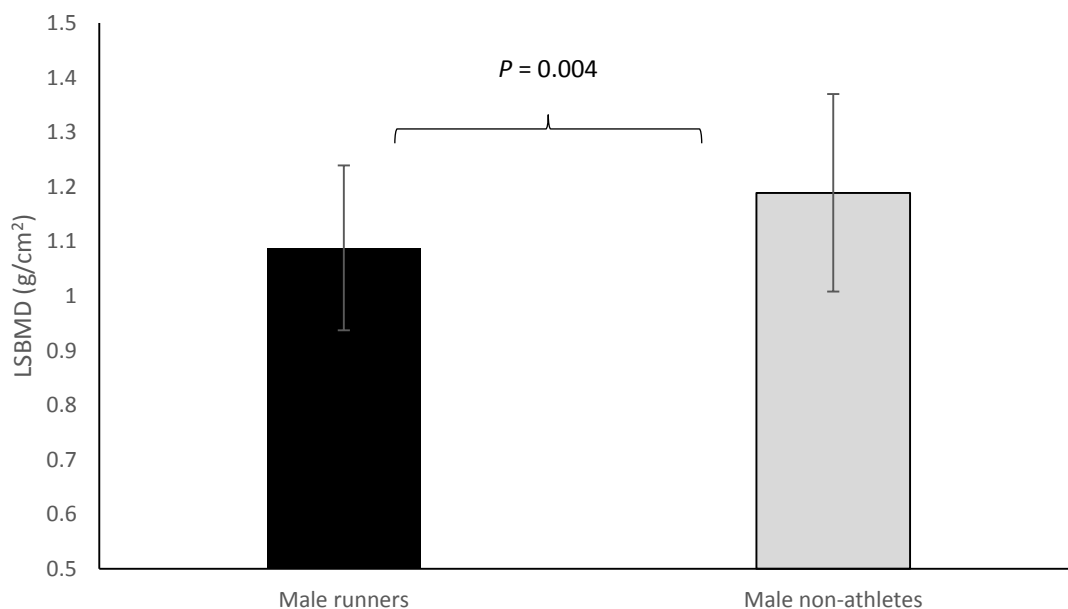
## *Statistical analysis*

Multiple analysis of variance (MANOVA) was used to compare TBMD, LBMD and LSBMD between the female runners and non-athletes and as well as between male runners and their non-athlete counterparts. To account for any potential influence of menstruation, the TBMD, LBMD and LSBMD of female runners who exhibited signs of amenorrhea were compared with those who were classed as eumenorrheic via

MANOVA analysis. Body mass-adjusted TBMD, LBMD, LSBMD, T-score and Z-score values were also reported and analysed via multiple analysis of covariance (ANCOVA). Alpha was set at 0.05 and data was reported as mean (SD) unless otherwise stated.

### 3.3 Results

LSBMD was <10% lower in male runners than non-athletes controls ( $P = 0.004$ ; Figure 3.1) but there were no differences in TBMD ( $P = 0.176$ ), LBMD ( $P = 0.963$ ), T-score ( $P = 0.123$ ) or Z-score ( $P = 0.092$ ) between these two groups (Table 3.1).



**Figure 3.1:** Difference in LSBMD between male runners and male non-athletes ( $P = 0.004$ ). Error bars denote SD.

**Table 3.1:** Anthropometric and unadjusted BMD phenotype data in male runners (n = 48) and non-athletes (n = 52). Data are presented as mean (SD) except for TBMD, LBMD, LSBMD, T-score and Z-score ranges which are presented as mean (minimum-maximum).

	Runners	Non-athletes
Age (years)	35 (9)	34 (14)
Height (m)	1.78 (0.06)	1.79 (0.07)
Mass (kg)	66.925 (6.606)	78.029 (10.752)
TBMD (g/cm <sup>2</sup> )	1.285 (0.094)	1.315 (0.114)
LBMD (g/cm <sup>2</sup> )	1.477 (0.108)	1.476 (0.138)
LSBMD (g/cm <sup>2</sup> )	<b>1.088 (0.151)*</b>	1.189 (0.181)
T-score	0.84 (0.88)	1.15 (1.07)
Z-score	0.82 (0.85)	1.13 (0.95)
TBMD Range	0.488 (1.034–1.522)	0.564 (1.067–1.631)
LBMD Range	0.526 (1.193–1.719)	0.749 (1.036–1.785)
LSBMD Range	0.810 (0.750–1.560)	0.820 (0.830–1.650)
T-score range	4.60 (-1.70–2.90)	5.10 (-1.40–3.70)
Z-score range	4.30 (-1.60–2.70)	4.70 (-0.90–3.80)

\* indicates difference from non-athletes.

Body-mass adjusted TBMD and LBMD were <4% and <6% higher, respectively, in male runners than non-athlete controls ( $P = 0.036$ ;  $P < 0.001$ ) but there were no differences in LSBMD ( $P = 0.345$ ), T-score ( $P = 0.111$ ) or Z-score ( $P = 0.106$ ) between the two groups (Table 3.2).

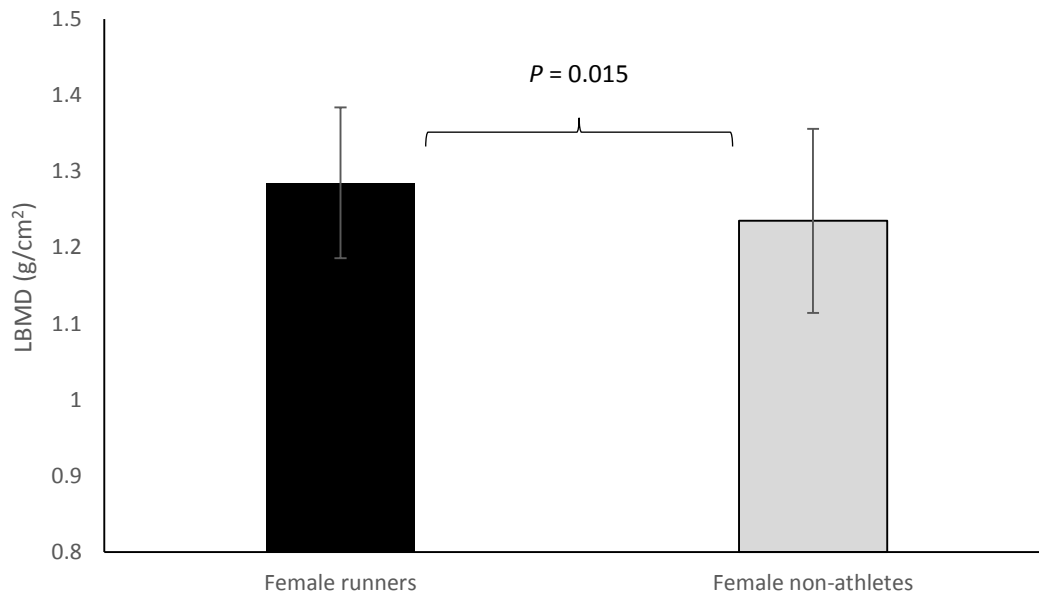
**Table 3.2:** Body mass-adjusted bone phenotype data in male runners (n = 48) and non-athletes (n = 52). Standard deviation (SD) is presented in brackets.

Group	Adj TBMD	Adj LBMD	Adj LSBMD	Adj T-score	Adj Z-score
Runners (n=45)	<b>1.325 (0.094)*</b>	<b>1.523 (0.107)*</b>	1.123 (0.174)	1.18 (0.94)	1.14 (0.80)
Non-athletes (n=52)	1.281 (0.094)	1.436 (0.108)	1.159 (0.173)	0.86 (0.94)	0.85 (0.79)
<i>P</i> - value	0.036	<0.001	0.345	0.111	0.106

\* indicates difference from non-athletes.



LBMD was ~4% higher in female runners than non-athlete controls ( $P = 0.015$ ; Figure 3.2) but there were no differences in TBMD ( $P = 0.508$ ), LSBMD ( $P = 0.110$ ), T-score ( $P = 0.475$ ) or Z-score ( $P = 0.847$ ) between these two groups (Table 3.3).



**Figure 3.2:** Difference in LBMD between female runners and female non-athletes ( $P = 0.015$ ). Error bar denotes SD.

**Table 3.3:** Anthropometric and unadjusted bone phenotype data in female runners ( $n = 58$ ) and non-athletes ( $n = 60$ ). Data are presented as mean (SD) except for TBMD, LBMD, LSBMD, T-score and Z-score ranges which are presented as mean (minimum-maximum).

	Runners	Non-athletes
Age (years)	34 (12)	37 (15)
Height (m)	1.65 (0.06)	1.64 (0.06)
Mass (kg)	52.934 (5.180)	64.659 (11.389)
TBMD (g/cm <sup>2</sup> )	1.203 (0.088)	1.191 (0.108)
LBMD (g/cm <sup>2</sup> )	<b>1.285 (0.099)*</b>	1.235 (0.121)
LSBMD (g/cm <sup>2</sup> )	1.127 (0.149)	1.175 (0.178)
T-score	1.15 (1.04)	1.00 (1.27)
Z-score	1.05 (0.90)	1.02 (1.15)
TBMD range	0.350 (1.010–1.360)	0.448 (0.991–1.439)
LBMD range	0.468 (1.029–1.497)	0.521 (0.993–1.513)
LS BMD range	0.760 (0.710–1.460)	0.700 (0.870–1.570)
T-score range	4.10 (-1.20–2.90)	5.30 (-1.50–3.80)
Z-score range	3.70 (-0.90–2.80)	4.30 (-0.80–3.50)

\* indicates difference from non-athletes.

No differences between TBMD ( $P = 0.293$ ), LBMD ( $P = 0.528$ ), LSBMD ( $P = 0.677$ ), T-score ( $P = 0.295$ ) or Z-score ( $P = 0.740$ ) were observed between amenorrheic and eumenorrheic runners (Table 3.4).

**Table 3.4:** Anthropometric and unadjusted bone phenotype data in amenorrheic (n = 18) and eumenorrheic (n = 40) runners. Standard deviation (SD) is presented in brackets.

	<b>Eumenorrheic</b>	<b>Amenorrheic</b>
Age (years)	37 (11)	27 (12)
Height (m)	1.64 (0.06)	1.66 (0.06)
Mass (kg)	52.899 (4.838)	53.011 (6.022)
TBMD (g/cm <sup>2</sup> )	1.211 (0.089)	1.185 (0.086)
LBMD (g/cm <sup>2</sup> )	1.290 (0.094)	1.272 (0.111)
LSBMD (g/cm <sup>2</sup> )	1.132 (0.146)	1.114 (0.158)
T-score	1.25 (1.04)	0.94 (1.02)
Z-score	1.08 (0.91)	0.99 (0.89)

Body mass-adjusted TBMD was ~5% and LBMD ~9% higher in the female runners compared to the non-athletes ( $P = 0.005$ ;  $P < 0.001$ ). Body mass-adjusted T-scores ( $P = 0.004$ ) and Z-scores ( $P = 0.008$ ) were also higher in the runners compared to non-athletes but no differences in body mass-adjusted LSBMD were observed between the two groups ( $P = 0.893$ ; Table 3.5).

**Table 3.5:** Body mass-adjusted bone phenotype data in female runners and non-athletes. Standard deviation (SD) is presented in brackets.

<b>Group</b>	<b>Adj TBMD</b>	<b>Adj LBMD</b>	<b>Adj LSBMD</b>	<b>Adj T-score</b>	<b>Adj Z-score</b>
Runners (n=58)	<b>1.226</b> <b>(0.099)*</b>	<b>1.316</b> <b>(0.114)*</b>	1.154 (0.175)	<b>1.43</b> <b>(1.22)*</b>	<b>1.33</b> <b>(1.07)*</b>
Non-athletes (n=60)	1.168 (0.101)	1.205 (0.108)	1.149 (0.178)	0.73 (1.24)	0.75 (1.08)
<i>P</i> - value	0.005	<0.001	0.893	0.004	0.008

\* indicates difference from non-athletes.

### 3.4 Discussion

The aim of this chapter was to investigate TBMD, LBMD, LSBMD, T-score and Z-score in high-level endurance runners in comparison to a non-athlete control group. This study is consistent with previous research suggesting that some endurance runners may possess higher site-specific BMD (Duncan et al., 2002) but lower non-loading site BMD (Hind et al., 2006) than non-athlete controls as highlighted in Figures 3.1 and 3.2. Higher LBMD but not TBMD or LSBMD was observed in female runners compared to non-athletes whilst lower LSBMD but no differences in TBMD or LBMD were present in the male comparison. No differences were present in T-score or Z-score between runners and controls in both male and female comparisons. When adjusted for body mass, TBMD, LBMD, T-score and Z-score was substantially higher in both the males and females in comparison to their non-athlete counterparts.

LBMD was 0.050 g/cm<sup>2</sup> higher in female runners than female non-athletes, highlighting the effects of site-specific mechanical loading on the lower extremity in endurance runners, which is congruent with some previous research (Duncan et al., 2002; Scofield and Hecht, 2012; Nevill et al., 2003; Brahm et al., 1997). Nevill et al. (2003) reported higher BMD of the legs in female endurance runners in comparison to upper body sites. They concluded that site-specific loading may enhance lower-body BMD (via a positive osteogenic effect) at the expense of bone mass of the upper-body sites. This may explain why no difference in TBMD or LSBMD between female runners and non-athletes was found in this chapter. Opposing findings to this chapter, reporting lower LSBMD in female endurance runners in comparison to controls, have primarily been observed in those who may have low energy availability and/or menstrual dysfunction (Pettersson et al., 1999; Barrack et al., 2008a; Scofield and Hecht, 2012). Reduced energy availability has been demonstrated to negatively impact bone

metabolism (through examination of bone turnover markers), consequently suppressing bone formation and potentially reducing BMD (Ihle and Loucks, 2004).

Reduced energy availability can be particularly harmful for BMD during childhood. Adolescent endurance runners have been reported to have lower BMD at sites such as the hip or the lumbar spine in both males and females (Barrack et al., 2008a; Barrack et al., 2017). Risk factors for low BMD in this population have included running over >50 km per week, being <85% of expected body mass and consuming <1 serving per day of a calcium-rich food (Barrack et al., 2017). In this chapter, no differences were observed in TBMD or LBMD between amenorrheic and eumenorrheic runners, suggesting that energy deficiency and menstrual status may not be influencing BMD in this investigated cohort, which may explain why no differences in TBMD were observed between female runners and non-athletes. It could be speculated that runners who experience energy deficiency and/or exercise-induced amenorrhea may find it more difficult to reach high-level due to the potential increased injury risk or negative effect on health from possessing lower BMD. Experiencing an injury has been shown to lead to cessation in sporting activities or reduction in participation, particularly in adolescents (Crane and Temple, 2015). It must be noted, however, that energy deficiency is difficult to measure and a self-report questionnaire was implemented to assess potential amenorrhea. Measurement error has been demonstrated in self-reported cycle length to assess menstruation status or history (Small et al., 2007) and thus, limitation exists when utilising this method. Questionnaire use, however, is inexpensive and easy to implement in large cohorts, and hence, has been widely accepted and used extensively to determine potential menstruation disturbances in athlete studies (Martin et al., 2017; Rosetta et al., 2001; Hoch et al., 2009).

Another possible explanation for the contradictory findings in this study (higher LBMD and no difference in TBMD) compared to previous investigations reporting low or lower

BMD may be due to the standard of the runners in the investigated cohort. This chapter utilised high-level runners who were all at a UK national standard for their respective PBs and completing large training distances between 55–215 km per week on average. Of the limited investigation into BMD of elite athletes, most studies have used non-elite athletes who are likely to complete lower volumes of training and consequently, be less at risk of energy deficiency and the associated negative effect on BMD. Although greater running distance per week has been negatively correlated with BMD (Hind et al., 2006; Burrows et al., 2003), higher-level runners are more likely to undertake resistance/strength based training, which may constitute greater amounts of high and multi-directional force to the bone, consequentially benefiting BMD (Nevill et al., 2003). Runners who complete higher volumes of resistance-based training have been found to possess higher LSBMD (Gordon and Nelson, 2003). The addition of high loading force on the bone via more strength-based training in the higher-level runners may explain why no difference in LSBMD between the female runners and controls was reported in this chapter. Pollock et al. (2010) studied a homogenous group of elite UK female endurance runners and is the only other study to date (alongside this investigation) that has studied a large cohort of UK endurance runners who are all competing at a high-level (i.e. nationally and/or internationally). Low TBMD (characterised by a Z-score of -1.0 to -2.0) was observed in 4.9% of the runners and a total-body Z-score median of approximately 0.1 was exhibited. In this chapter, a total-body Z-score of -0.9 was the lowest observed in the female runners and a mean TBMD of 1.053 (g/cm<sup>2</sup>) was found, suggesting a higher TBMD in the runners investigated in this chapter. Although the runners utilised in this and the Pollock et al. (2010) study were of a similar standard, the practices and undertaking of strength or resistance-based training by endurance runners, however, varies substantially (Blagrove et al., 2017), which may explain the different findings between the two investigations.

Male runners exhibited lower LSBMD compared to non-athlete counterparts, which is congruent with some previous research. Lower vertebral but not tibial or radial BMD has been observed in male endurance runners completing  $92.2 \pm 6.3$  km per week (Bilanin et al., 1989), whilst Hind et al. (2006) reported low lumbar spine BMD in comparison to a reference population in both female and male endurance runners. Endurance runners tend to have lower body mass than non-athlete controls, as observed in this chapter, and thus less force during loading will be exerted on these anatomical sites in comparison to non-athletes. Furthermore, the lumbar spine experiences less mechanical loading than the lower extremity when running which may also explain why lower BMD at this site in comparison to the non-athletes is present (Pollock et al., 2010; Cappozzo, 1983).

It is surprising to find no difference in LBMD between the male runners and non-athletes given that up to 10% higher LBMD in runners compared to non-athletes has been reported in a previous investigation (Stewart and Hannan, 2000), although similar TBMD and LBMD to non-athletes has also been reported previously (Fredericson et al., 2007). Lower LSBMD and no differences in TBMD or LBMD in the runners in comparison to the non-athletes found in this chapter may be due to the potential effect of RED-S. Previous investigations suggest that male runners may be at risk of RED-S (Tenforde et al., 2016; Barrack et al., 2017) as highlighted in the recent IOC consensus statement (Mountjoy et al., 2018). The benefits of mechanical loading on BMD, therefore, can sometimes be overcome or reduced by the presence of an energy deficiency. Essentially, the balance of bone turnover following repeated training in male endurance runners does not appear to be affected unless an energy deficiency is present, resulting in suppression of bone formation (Zanker and Swaine, 2000). A greater magnitude of loading at the lower extremity and the associated mechanical impact from running may protect against the potential negative effect of reduced

energy availability on BMD and consequently explain why lower LSBMD but similar TBMD and LBMD was found in comparison to the control group. Varying levels of energy availability and genetic profiles may explain the differences in results in the literature and why lower or similar BMD in comparison to non-athletes has been observed in endurance running populations.

Total-body T-scores and Z-scores were generated from the manufacturer's reference system (National Health and Nutrition Examination Survey, 2008). No differences were observed in total-body T-score and Z-score between runners in both the male and female analysis as would be expected due to non-significant findings in the comparison of TBMD. The runners had positive mean total-body T- and Z-scores (males = 0.842, 0.816; females = 1.153, 1.053), which contradicts some previous literature reporting low total-body T- and/or Z-scores in endurance running populations (Pollock et al., 2010). It must be noted, however, that comparison across studies is difficult due to the different DXA machines utilised and different reference databases used to compare BMD data. The T-scores and Z-scores for both the runners and non-athletes were substantially higher than this reference database, suggesting that the mechanical loading completed by the runners has been beneficial for TBMD and that the non-athlete group may have been completing enough physical activity to initiate an osteogenic response although deemed non-athletes. It would be interesting to obtain T-scores and Z-scores for the other measured sites (LBMD and LSBMD). Comparing the BMD for these sites to a large reference database would help identify if the LSBMD of the male runners would be interpreted as low and the LBMD of the female runners considered high in comparison to the norm. LSBMD that is classed as low or osteoporotic has been observed in endurance runners in comparison to norms previously (Pollock et al., 2010; Hind et al., 2006).

It is interesting to note the large variance in TBMD, LBMD and LSBMD in both the control group and the endurance runners. This substantial range cannot be attributed solely to age- or physical activity associated effects on BMD and indicates that other determinants, such as genetics, significantly influence BMD. Heritability is suggested to be between 50-85% (Ralston and Uitterlinden, 2010) and a large number of genes have been hypothesised to influence BMD (Golchin et al., 2016; Hsu and Kiel, 2012). The control group possessed a larger range in both TBMD and LBMD in males and females than the runners, which suggests that runners could compensate for a potential disadvantageous genetic predisposition for BMD via mechanical loading as has been proposed in previous investigation (Mitchell et al., 2016).

This chapter suggests that female runners possess ~4% higher LBMD but not TBMD or LSBMD than female non-athletes, whereas male runners possess ~10% lower LSBMD but similar TBMD and LBMD compared to controls. Additionally, no differences were observed in total-body T- and Z-scores for male or female comparisons. This would suggest that female but not male runners benefit from site-specific mechanical loading on the skeleton, resulting in higher leg BMD. Male runners, however, appear to display lower BMD at the lumbar spine in comparison with non-athletes, which may be due to reduced energy availability. Additionally, a large range in BMD was observed in both the male and female runners and controls, suggesting that other determinants such as genetics, diet, types of loading and when this loading may have occurred also influences BMD across the adult lifespan.



## **Chapter 4:**

# **Association of bone mineral density-related genes with athlete status and marathon performance in high-level endurance runners**

## 4.1 Introduction

Bone mineral density (BMD) is considered the primary predictor of osteoporotic and fracture risk, and is heavily influenced by factors such as smoking (Law and Hackshaw, 1997), dietary intake (Darling et al., 2009) and physical activity (Pluijm et al., 2001; Krall and Dawson-Hughes, 1993). Following bouts of physical activity, osteocytes detect shape and volume changes to increase or decrease the liberation of bone mediators, which consequently influences bone formation and resorption (Nakashima et al., 2011). Thus, completing a larger volume of physical activity/exercise across the lifespan increases BMD, reportedly accounting for up to 30% of the variability in total BMD (Valdimarsson et al., 1999; Neville et al., 2002; Allison et al., 2015). Athletes who compete in weight-bearing sports, therefore, tend to possess higher BMD than non-athletes, due to the increased strain rates and high peak-force loading characteristics experienced by this mechanical loading (Fehling et al., 1995). Despite this, too much activity to the point of overtraining can result in negative outcomes (Kuipers and Keizer, 1988), such as stress fractures (Knapp and Garrett, 1997), which occur due to the repetitive mechanical loading that stimulates an incomplete remodelling response (Jones et al., 2002). Higher incidence of lower limb stress fractures is observed in endurance runners in comparison with non-athletic controls. Significant amounts of site-specific loading combined with other factors typical of this group, such as low energy availability, can result in lower BMD and a higher risk of fracture occurrence (Loucks, 2007).

Stress fractures have been reported to account for 50% of all injuries sustained by runners and military recruits, with higher incidence observed in females, which may be due to other influential factors such as lower lean mass, leg-length discrepancy and irregular menstruation (Milner et al., 2006). Interestingly, in both female and male runners with a history of stress fracture, lower BMD has been observed (Bennell et al.,

1996; Barrack et al., 2017). Consequently, higher BMD appears important for elite endurance running to allow athletes to complete larger volumes of training with less potential training interruption from injury, which in turn may increase their likelihood of achieving success.

Although BMD is a multi-factorial phenotype, influenced by diet, hormones and physical activity, heritability is suggested to be between 50-85% depending upon anatomical location (Ralston and Uitterlinden, 2010). To date, nearly 100 loci have been associated with BMD and/or osteoporosis via GWAS (Rocha-Braz and Ferraz-de-Souza, 2016). Similarly, stress fracture occurrence has been reported to have a genetic influence in both military recruits (Korvala et al., 2010; Yanovich et al., 2011) and athletic populations (Varley et al., 2015; Varley et al., 2016; Varley et al., 2017). Many of the variants associated with BMD and/or stress fracture, however, have not been replicated in further study or investigated in different populations where the outcome of gene-environment interactions can be explored. The lack of replication is particularly evident in those who may exhibit extreme phenotypes or genotypes, or undertake a substantial volume of mechanical loading, such as elite marathon runners, who have been reported to run an average minimum weekly distance of 150 km ( $\pm$  17 km) (Billat et al., 2001).

Greater understanding of the BMD-associated genetic profile in endurance athletes may further our understanding of the complex phenotype of successful endurance performance and provide new insight into the genetic association with BMD. The primary aim of this study, therefore, was to compare the genotype and allele frequencies of 10 genetic variants (detailed in Section 1.5 - *AXIN1* rs9921222, *BDNF-AS* rs6265, *COL1A1* rs1800012, *COMT* rs4680, *LRP5* rs3736228, *P2RX7* rs3751143, *TNFRSF11A* rs3018362, *TNFRSF11B* rs4355801, *VDR* s2228570, *WNT16* rs3801387) associated with BMD in high-level endurance runners (elite and sub-elite)

and a non-athlete control group. Additionally, any association with marathon PB was explored for each variant individually, and collectively, as part of a Total Genotype Score (TGS) before receiver operator curve (ROC) area under the curve (AUC) analysis was implemented to determine if TGS could distinguish runners from non-athletes. It was hypothesised that the runners, particularly those in the elite category, would possess fewer “risk” (lower BMD-associated) genotypes than non-athlete controls that would contribute to a potential reduced risk of injury, resulting in less training interruption and greater competitive success.

## **4.2 Method**

The investigated participants and protocols used in Chapter 4 have already been described in detail in Chapter 2, thus, only a brief description of these methods is detailed below.

### *Participant characteristics*

Participants consisted of 528 European Caucasian marathon runners and 559 ethnically matched non-athlete controls from the cohort as described in Table 2.1 of Chapter 2. The marathon runners were sub-grouped by personal best marathon (PB) time into either elite (men < 2 h 30 min, n = 128; women < 3 h 00 min, n = 119) or sub-elite (men 2 h 30 min – 2 h 45 min, n = 196; women 3 h 00 min – 3 h 15 min, n = 85) categories.

### *Procedure*

All participants provided a whole-blood, saliva or buccal swab sample, from which DNA was subsequently extracted and analysed to obtain genotype data for the 10 investigated SNPs as described in Chapter 2.

### *Statistical analysis*

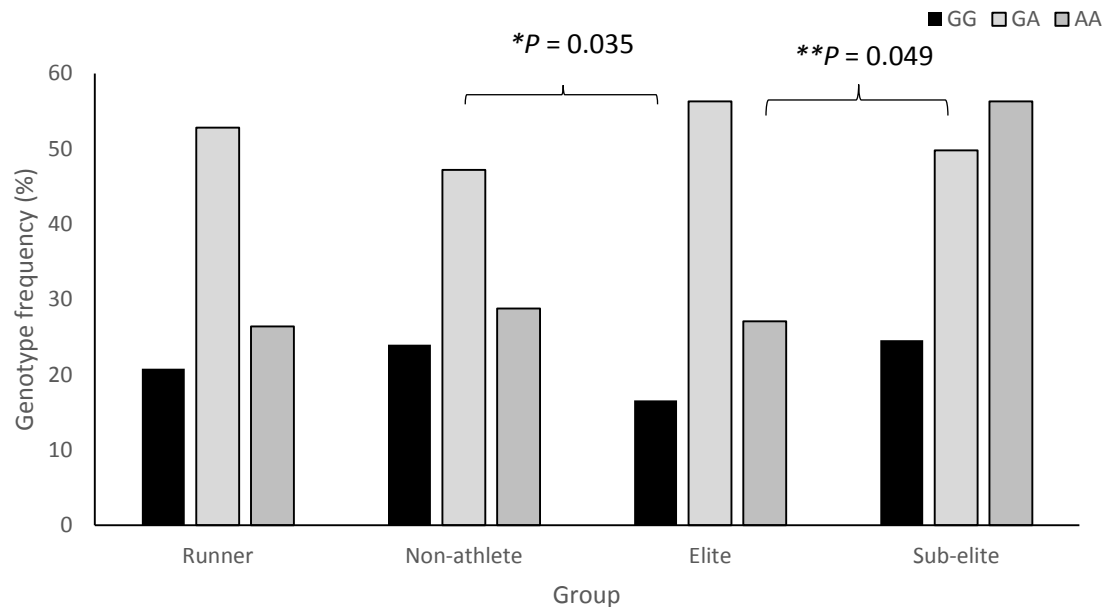
Pearson's Chi-square ( $\chi^2$ ) tests were utilised to compare genotype (using three analysis models; additive, recessive and dominant) and allele frequencies between all runners and non-athletes as well as elite vs non-athletes before completing an elite vs sub-elite and then sub-elite vs non-athlete comparison if an initial association was observed. Odds ratios were also implemented to estimate effect size. One-way analysis of variance (ANOVA) was used to determine if PB differed between genotype groups for all 10 variants, individually and collectively as a TGS, whilst linear regression was also implemented to evaluate whether TGS could predict marathon PB. TGS was calculated using the model reported in Williams and Folland (2008), and thus, every SNP was allocated a score according to existing literature. Each SNP homozygote associated with higher BMD was given a score of 2, the heterozygotes scoring 1 and the other homozygote given 0 ("risk" genotype). The total score was then calculated to lie within 0-100 ((e.g. TGS =  $100/20 \times (2+1+0+1+1+1+0+2+1+2) = 55$ )). Independent t-test analyses were completed to investigate any differences between runners and non-athletes, elite and sub-elite athletes (total and according to sex, i.e. elite men vs sub-elite men and elite women vs sub-elite women) and the fastest 10% compared to the slowest 10% allocated by PB (both collectively and sex-dependent). Receiver operator curve (ROC) area under the curve (AUC) analysis was conducted to determine if the TGS was able to classify runners from non-athletes. Benjamini-Hochberg corrections were implemented for genotype and allele frequency comparisons when required (each variant submitted to 8 - 9 tests) to account for false discovery rate. Corrected probability values are reported (unless stated) and alpha was set at 0.05.

### 4.3 Results

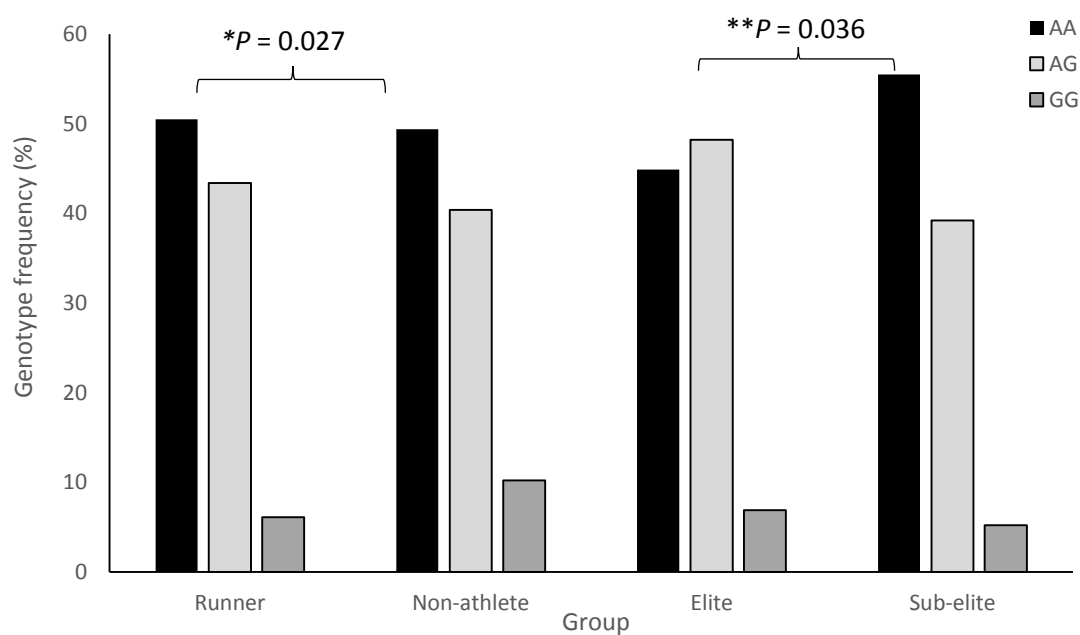
#### *Genotype frequencies*

All genotype data for runners and non-athletes were in Hardy-Weinberg equilibrium ( $P \geq 0.060$ ,  $\chi^2 = 3.524$ ) except for the *VDR* rs2228570 genotype in the control group ( $P = 0.035$ ,  $\chi^2 = 4.461$ ). For *COMT* rs4680, the elite group possessed a lower frequency (~8%) of the “risk” (lower BMD-associated) GG genotypes than the non-athletes ( $P = 0.035$ ,  $\chi^2 = 10.123$ ; Figure 4.1) and sub-elite groups ( $P = 0.049$ ,  $\chi^2 = 8.659$ ; Figure 4.1) with a trend observed between the runner and non-athlete group ( $P = 0.083$ ,  $\chi^2 = 6.816$ ). Similarly, in the recessive analysis model, a difference between the genotype frequencies of the elite runners compared to non-athletes as well as the sub-elite group was observed ( $P = 0.035$ ,  $\chi^2 = 7.366$ ;  $P = 0.035$ ,  $\chi^2 = 8.439$ ). For *WNT16* rs3801387, genotype frequency differed between the runner and non-athlete groups ( $P = 0.027$ ,  $\chi^2 = 10.141$ ) with runners possessing a ~4% lower frequency of the advantageous GG genotype. Elite runners possessed an 11% lower frequency of the “risk” AA genotype in comparison to sub-elite ( $P = 0.036$ ,  $\chi^2 = 6.816$ ) and a trend for a lower frequency (4%) was observed in comparison to non-athletes ( $P = 0.059$ ,  $\chi^2 = 7.313$ ; Figure 4.2). In the dominant analysis model, runners exhibited a larger number of “risk” A carriers in comparison to non-athletes ( $P = 0.018$ ,  $\chi^2 = 9.865$ ). Genotype frequency differed between the runner and non-athlete groups for both the additive and recessive analysis models ( $P = 0.027$ ,  $\chi^2 = 9.199$ ;  $P = 0.012$ ,  $\chi^2 = 8.834$ ; Figure 4.3) for the *P2RX7* rs3751143 variant but no differences were observed for the elite vs non-athlete comparisons ( $P \geq 0.162$ ,  $\chi^2 = 3.091$ ). Genotype frequency differed before multiple testing correction between the runner and non-athlete group in the *VDR* rs2228570 variant for both the additive and dominant analysis model with runners possessing a <5% fewer frequency of “risk” T carriers ( $P = 0.180$ ,  $\chi^2 = 7.287$ ;  $P = 0.180$ ,  $\chi^2 = 7.287$ ;

Figure 4.3). No differences were observed for any other investigated variant in runner vs non-athlete or elite vs non-athlete comparisons ( $P \geq 0.330$ ; Table 4.1).

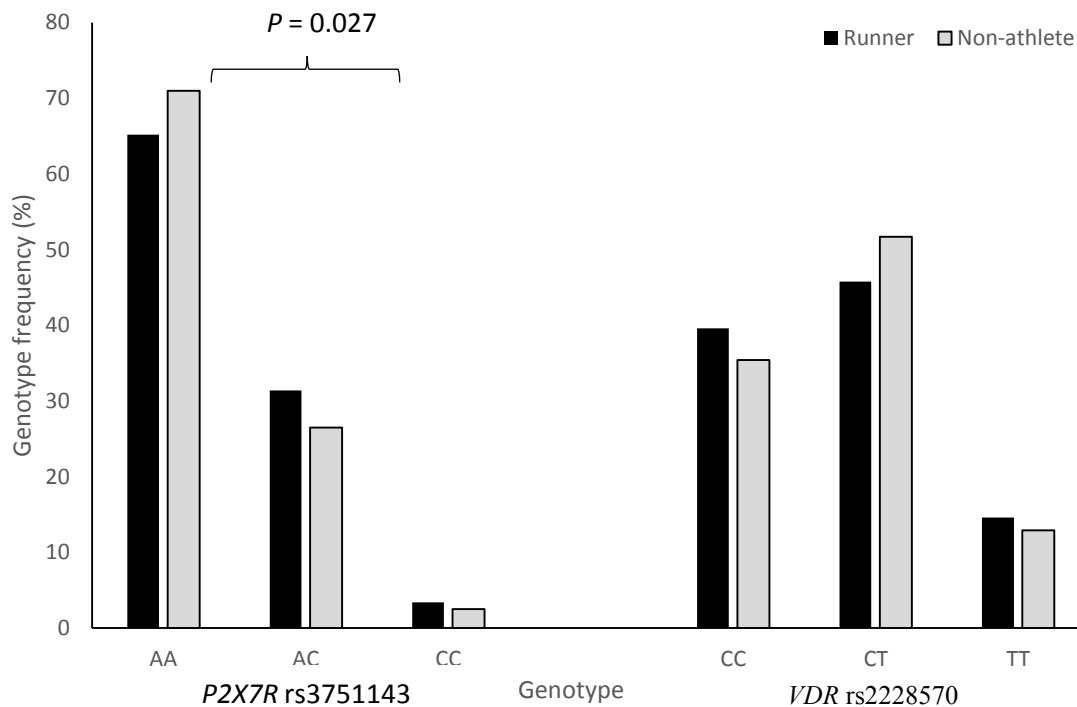


**Figure 4.1:** *COMT* rs4680 genotype frequencies of runners and non-athletes, with runners divided into standard subgroups (elite and sub-elite). Differences between elite vs non-athlete (\* $P = 0.035$ ,  $\chi^2 = 10.123$ ) and elite vs sub-elite comparisons (\*\* $P = 0.049$ ,  $\chi^2 = 8.649$ ).



**Figure 4.2:** *WNT16* rs3801387 genotype frequencies of runners and non-athletes, with runners divided into standard subgroups (elite and sub-elite). Significant differences

between runners and non-athletes ( $*P = 0.027$ ,  $\chi^2 = 10.141$ ) and elite vs sub-elite ( $**P = 0.036$ ,  $\chi^2 = 11.229$ ) comparisons.

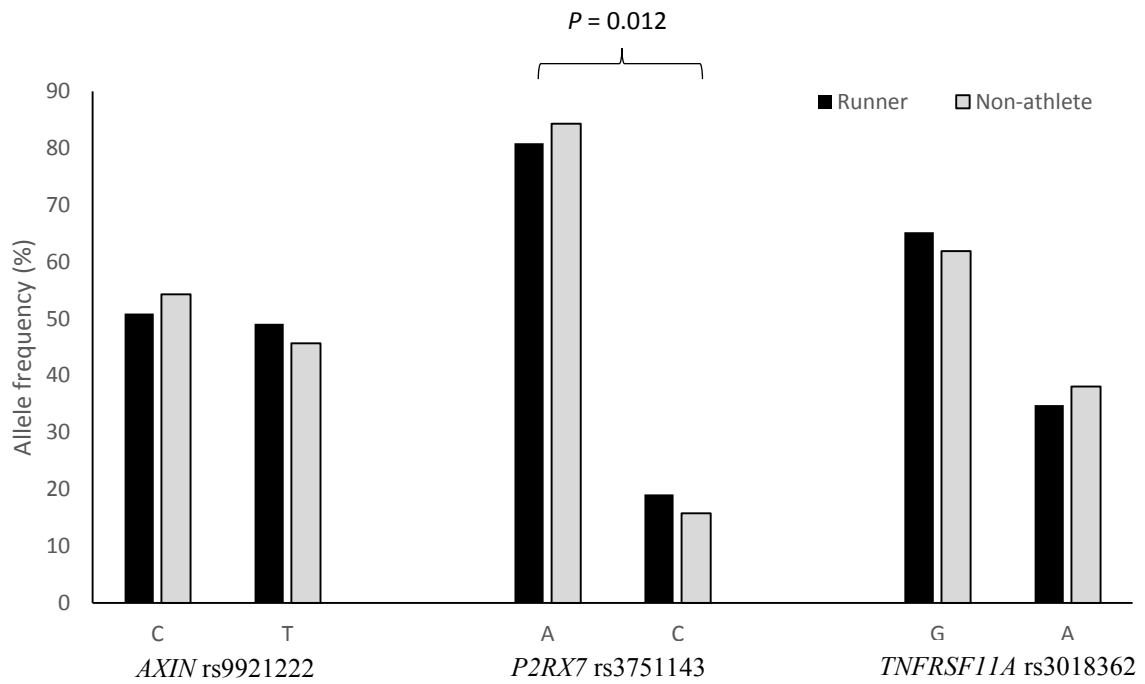


**Figure 4.3:** *P2RX7* rs3751143 and *VDR* rs2228570 genotype frequencies of runners and non-athletes groups. Difference in *P2RX7* rs3751143 genotype frequency between runners and non-athletes ( $P = 0.027$ ,  $\chi^2 = 9.199$ ).

#### Allele frequencies

Runners possessed a 4% higher frequency of the *P2RX7* rs3751143 “risk” C allele than non-athletes ( $P = 0.012$ ,  $\chi^2 = 9.130$ ) but no difference was observed in the elite vs non-athlete comparison ( $P = 0.158$ ; Figure 4.4). Allele frequency also differed between runners and non-athletes for the *AXIN1* rs9921222 and *TNFRSF11A* rs3018362 variants but not after multiple testing correction ( $P = 0.112$ ,  $\chi^2 = 5.039$ ;  $P = 0.116$ ,  $\chi^2 = 4.745$ ; Figure 4.4). No differences in allele frequency were observed for any other variant in runners vs non-athletes and elite vs non-athletes ( $P \geq 0.108$ ; Table 4.1).





**Figure 4.4:** *AXIN1* rs9921222, *P2RX7* rs3751143 and *TNFRSF11A* rs3018362 allele frequencies of runners and non-athletes. Difference in *P2RX7* rs3751143 allele frequency between runners and non-athletes ( $P = 0.012$ ,  $\chi^2 = 9.130$ ).

**Table 4.1:** Genotype and allele frequencies of all investigated variants in runners and non-athletes with their respective significant\* or non-significant *P*- and  $\chi^2$  values.

SNP	Genotypes Alleles	Groups			Group comparison	
		Runners	Non-athletes	Elite	Runner vs Non-athlete P-value	Elite vs Non- athlete P-value
		Genotype frequency (%)	Genotype frequency (%)	Genotype frequency (%)		
		Allele frequency (%)	Allele frequency (%)	Allele frequency (%)	Chi-square ( $\chi^2$ )	Chi-square ( $\chi^2$ )
<i>AXIN1</i> rs9921222	CC	26.3	29.3	31.2	0.168	0.546
	CT	49.1	49.9	45.7	(5.521)	(1.785)
	TT	24.6	20.8	23.1		
	C	50.9	54.3	44.7	0.112	0.913
	T	49.1	45.7	55.3	(5.039)	(0.012)
<i>BDNF-AS</i> rs6265	GG	66.1	67.8	66.4	0.704	0.808
	GA	30.3	28.8	29.6	(0.701)	(0.427)
	AA	3.6	3.4	4.0		
	G	81.2	82.2	81.2	0.419	0.551
	A	18.8	17.8	18.8	(0.652)	(0.356)
<i>COL1A1</i> rs1800012	AA	65.0	68.0	64.1	0.330	0.782
	AC	31.4	28.8	32.4	(2.217)	(0.491)
	CC	3.6	3.2	3.6		
	A	80.7	82.4	81.2	0.148	0.482
	C	19.3	17.6	18.8	(2.096)	(0.944)
<i>COMT</i> rs4680	GG	20.8	24.0	16.6	0.083	<b>0.035*</b>
	GA	52.8	47.2	56.3	(6.816)	(10.123)
	AA	26.4	28.8	27.1		
	G	47.3	47.6	44.7	0.829	0.205
	A	52.7	52.4	55.3	(0.046)	(1.607)
<i>LRP5</i> rs3736228	GG	73.7	73.0	64.1	0.663	0.429
	GA	24.4	24.5	32.4	(0.821)	(1.693)
	AA	1.9	2.5	3.6		
	G	85.9	85.2	86.2	0.552	0.534
	A	14.1	14.8	13.8	(0.353)	(0.387)
<i>P2RX7</i> rs3751143	AA	65.2	71.0	66.8	<b>0.027*</b>	0.216
	AC	31.4	26.5	29.1	(9.199)	(3.637)
	CC	3.4	2.5	4.1		
	A	80.9	84.3	81.4	<b>0.012*</b>	0.158
	C	19.1	15.7	18.6	(9.130)	(3.091)
<i>TNFRSF11A</i> rs3018362	GG	42.8	37.9	41.3	0.065	0.410
	GA	44.7	50.0	43.7	(5.469)	(1.782)
	AA	12.5	14.1	15.0		
	G	65.2	61.9	63.2	0.116	0.564
	A	34.8	38.1	36.8	(4.745)	(0.373)
<i>TNFRSF11B</i> rs4355801	AA	30.3	28.1	30.4	0.507	0.725
	GA	50.0	52.1	50.2	(1.360)	(0.642)
	GG	19.7	19.9	19.4		
	A	55.3	54.1	55.5	0.438	0.547
	G	44.7	45.9	45.5	(0.601)	(0.363)
<i>VDR</i> rs2228570	CC	39.6	35.4	40.1	0.180	0.272
	CT	45.8	71.7	45.3	(7.287)	(3.995)
	TT	14.6	12.9	14.6		
	C	62.5	61.3	62.8	0.499	0.499
	T	37.5	38.7	37.2	(0.673)	(0.458)
<i>WNT16</i> rs3801387	AA	50.6	49.4	44.9	<b>0.027*</b>	0.059
	AG	43.3	40.4	48.2	(10.141)	(7.313)
	GG	6.1	10.2	6.9		
	A	72.3	69.6	69.0	0.108	0.787
	G	27.7	30.4	31.0	(3.545)	(0.073)

**Table 4.2:** Odds ratio and confidence interval (CI) statistics for runner vs non-athlete status of the 10 investigated SNPs.

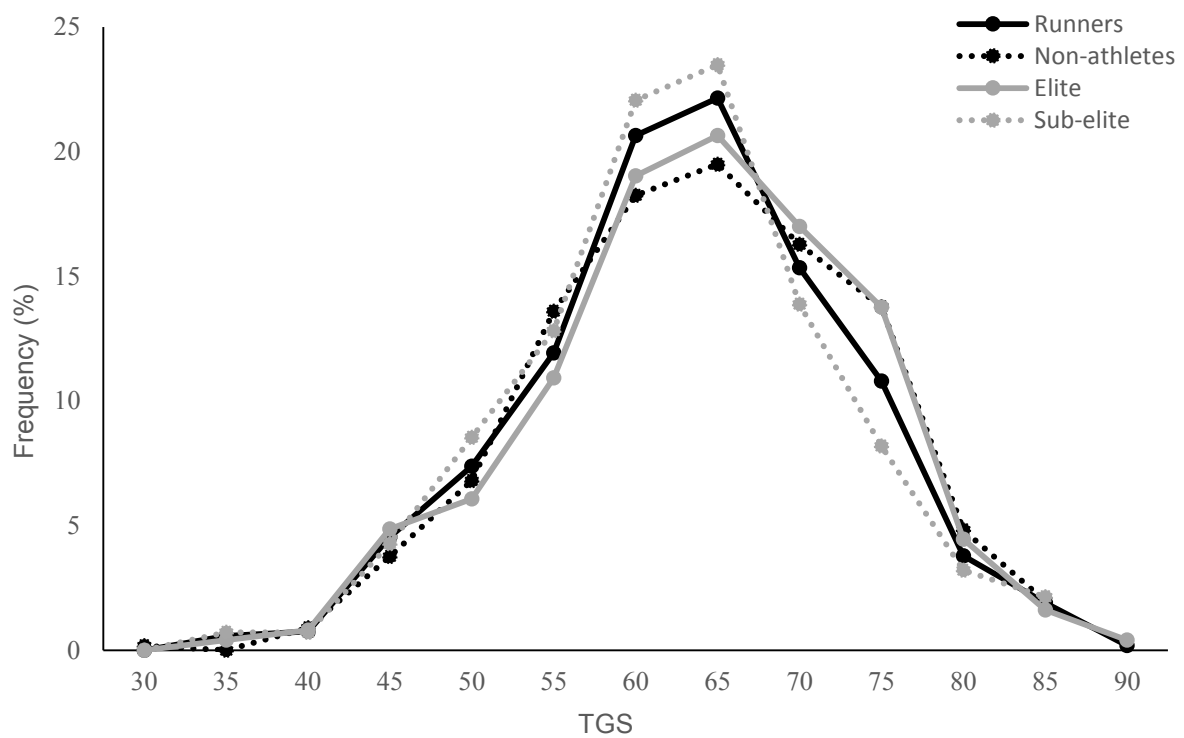
SNP	Genetic model	Odds ratio Runner vs NA	95% CI	Odds ratio Elite vs NA	95% CI
<i>AXIN1</i> rs9921222	T/C	1.15	0.970 – 1.359	1.14	0.918 – 1.148
	TT/CC	1.26	0.832 – 1.918	1.05	0.690 – 1.588
	TT/C carriers	1.25	0.938 – 1.658	1.15	0.800 – 1.642
	T carriers/CC	1.16	0.891 – 1.516	0.92	0.662 – 1.269
<i>BDNF-AS</i> rs6265	A/G	1.07	0.857 – 1.325	1.07	0.815 – 1.407
	AA/GG	1.09	0.566 – 2.085	1.22	0.553 – 2.673
	AA/G carriers	1.06	0.555 – 2.027	1.20	0.549 – 2.618
	A carriers/GG	1.08	0.839 – 1.391	0.88	0.390 – 1.967
<i>COL1A1</i> rs1800012	C/A	1.12	0.901 – 1.390	1.08	0.825 – 1.425
	CC/AA	1.17	0.604 – 2.265	1.17	0.513 – 2.649
	C/A carriers	1.12	0.582 – 2.162	1.14	0.503 – 2.567
	C carriers/AA	1.15	0.890 – 1.473	1.09	0.796 – 1.503
<i>COMT</i> rs4680	G/A	0.99	0.834 – 1.168	0.89	0.721 – 1.103
	GG/AA	0.95	0.677 – 1.335	0.74	0.468 – 1.154
	GG/A carriers	0.84	0.627 – 1.111	0.63	0.429 – 0.930
	G carriers/AA	1.13	0.867 – 1.478	1.09	0.777 – 1.519
<i>LRP5</i> rs3736228	A/G	0.95	0.747 – 1.206	0.92	0.680 – 1.250
	AA/GG	0.75	0.329 – 1.707	0.48	0.136 – 1.692
	AA/G carriers	0.75	0.331 – 1.707	0.48	0.136 – 1.681
	A carriers/GG	0.97	0.738 – 1.264	0.97	0.687 – 1.355
<i>P2RX7</i> rs3751143	C/A	1.23	1.014 – 1.581	1.23	0.928 – 1.617
	CC/AA	1.48	0.727 – 3.028	1.72	0.748 – 3.941
	CC/A carriers	1.37	0.676 – 2.791	1.64	0.719 – 3.751
	C carriers/AA	1.40	1.078 – 1.810	1.30	0.939 – 1.795
<i>TNFRSF11A</i> rs3018362	A/G	0.87	0.729 – 1.035	0.95	0.761 – 1.180
	AA/GG	0.78	0.538 – 1.142	0.97	0.617 – 1.536
	AA/G carriers	0.87	0.611 – 1.233	1.07	0.701 – 1.634
	A carriers/GG	0.82	0.640 – 1.041	0.87	0.640 – 1.179
<i>TNFRSF11B</i> rs4355801	A/G	1.05	0.886 – 1.242	1.06	0.854 – 1.307
	AA/GG	1.09	0.769 – 1.538	1.11	0.714 – 1.709
	AA/G carriers	1.11	0.857 – 1.446	1.12	0.804 – 1.550
	A carriers/GG	1.01	0.749 – 1.362	1.03	0.704 – 1.498
<i>VDR</i> rs2228570	T/C	0.95	0.798 – 1.129	0.94	0.755 – 1.168
	TT/CC	1.01	0.696 – 1.475	1.00	0.627 – 1.596
	TT/C carriers	1.16	0.817 – 1.632	1.15	0.750 – 1.777
	T carriers/CC	0.84	0.655 – 1.071	0.82	0.603 – 1.116
<i>WNT16</i> rs3801387	A/G	1.14	0.945 – 1.370	0.97	0.774 – 1.225
	AA/GG	1.72	1.083 – 2.742	1.35	0.751 – 2.420
	AA/G carriers	1.05	0.827 – 1.331	0.83	0.615 – 1.122
	A carriers/GG	1.76	1.122 – 2.761	1.54	0.874 – 2.699

### Total Genotype Score

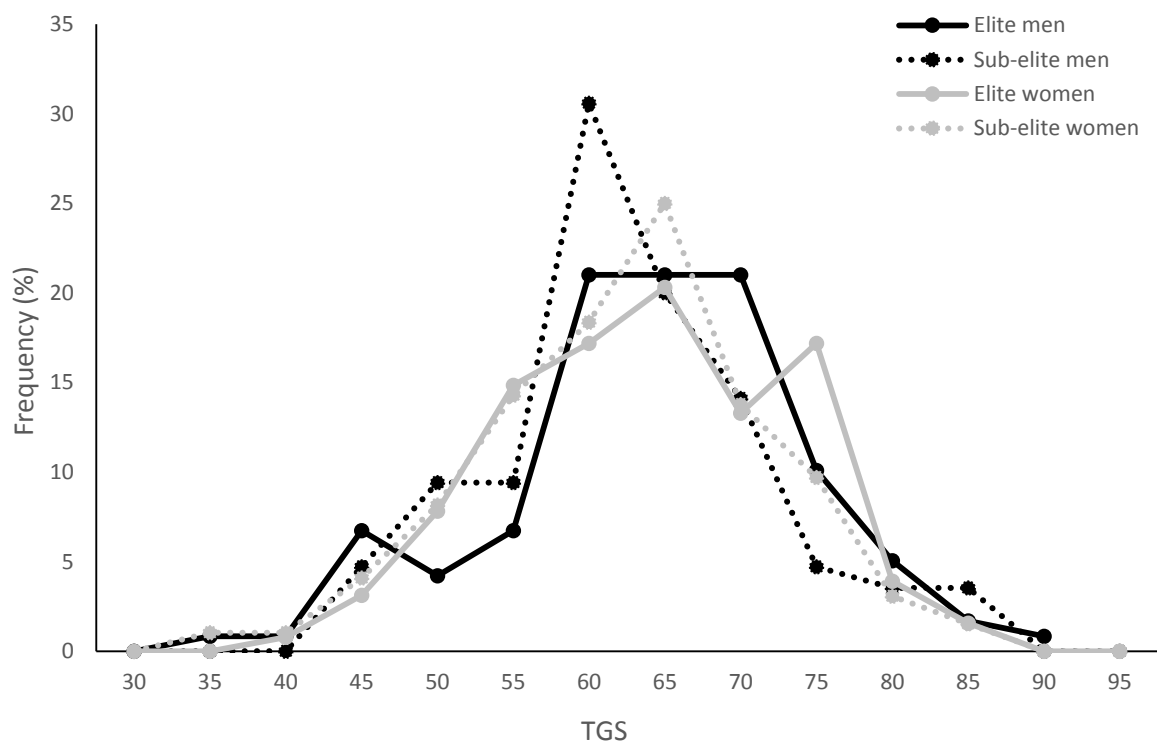
No differences were observed in TGS between athletes and controls ( $P = 0.188$ ) or any other comparison ( $P = 0.067$ ), with all subgroups displaying similar mean TGS (Table 4.3). ROC analysis determined that TGS frequency distribution could not distinguish a runner from a non-athlete ( $AUC = 0.477$ ,  $P = 0.192$ ). Additionally, ROC analysis could not differentiate between the top 10% and bottom 10% of male runners ( $P = 0.939$ ,  $AUC = 0.494$ ) but a tendency for a higher TGS frequency in the 10% compared to the bottom 10% in the female runners was observed ( $P = 0.053$   $AUC = 0.675$ ; Figure 4.6d).

**Table 4.3:** Mean, mode and median TGS of runners and non-athletes with runners divided into standard subgroups (elite and sub-elite) as well as the top 10% according to PB (corrected  $P$  – value).

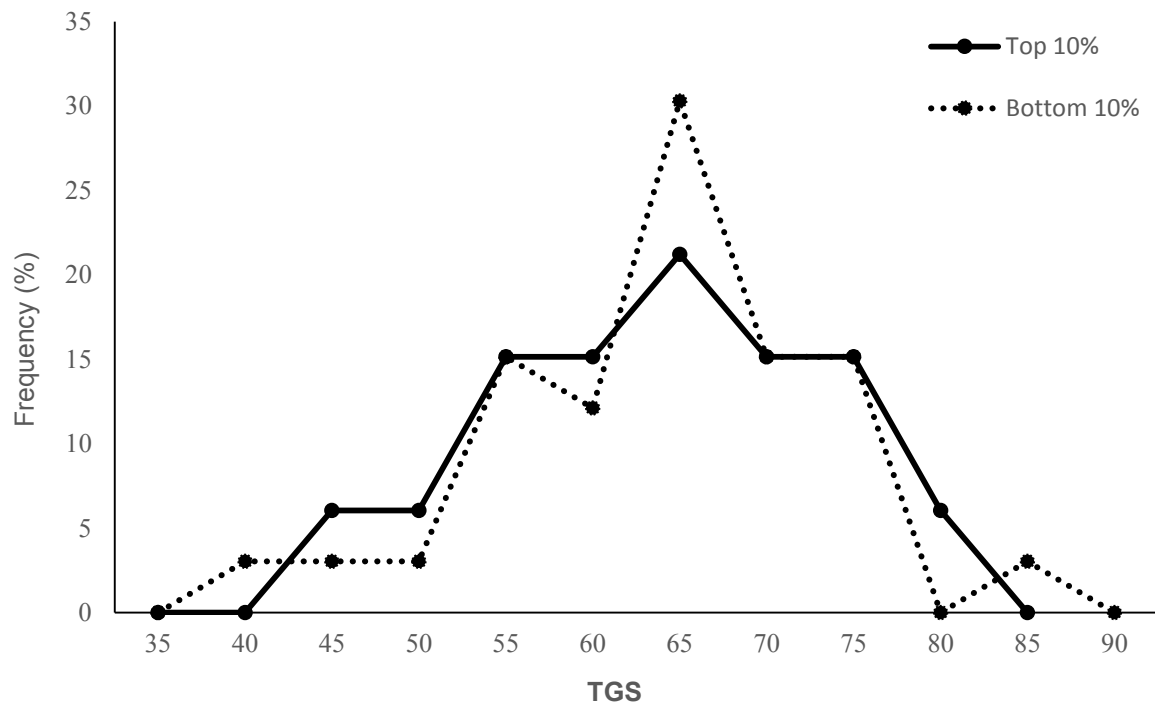
Group and comparisons	TGS Mean (SD)	Mode	Median	$P$ - value
Runners	63.23 (9.54)	65	65	0.188 (0.372)
Controls	64.03 (9.65)	65	65	
Elite	64.07 (9.70)	65	65	0.067 (0.372)
Sub-elite	62.54 (9.35)	65	65	
Elite men	63.95 (9.45)	65	65	0.188 (0.372)
Sub-elite men	62.52 (9.70)	65	65	
Elite women	64.20 (10.00)	60	65	0.239 (0.372)
Sub-elite women	62.59 (9.08)	60	60	
Top 10% all	63.89 (9.40)	70	65	0.533 (0.622)
Bottom 10% all	62.78 (9.04)	65	65	
Top 10% men	63.79 (9.52)	65	65	0.948 (0.948)
Bottom 10% men	63.94 (9.41)	65	65	
Top 10% women	64.05 (9.43)	70	65	0.266 (0.372)
Bottom 10% women	60.95 (8.31)	60	60	



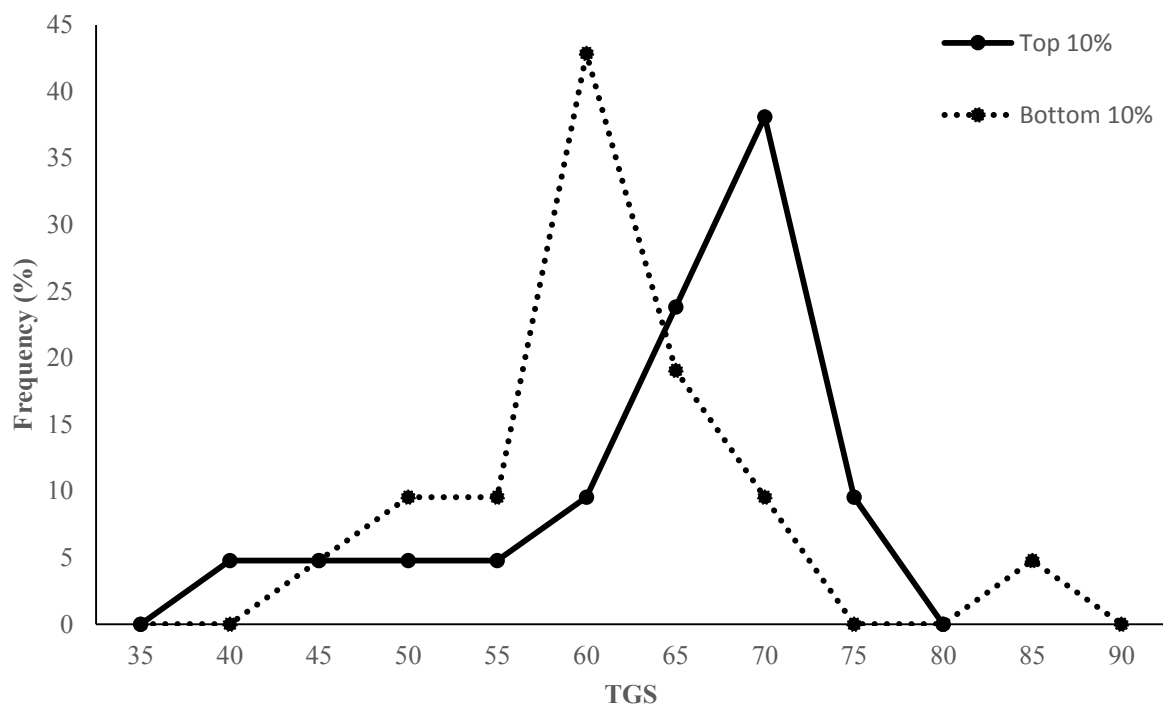
**Figure 4.6a:** TGS frequency distribution in male runners and non-athletes, with runners divided into elite and sub-elite groups.



**Figure 4.6b:** TGS frequency distribution in elite and sub-elite male and female runners.



**Figure 4.6c:** TGS frequency distribution in the elite, sub-elite, top 10% and bottom 10% PB male runners.



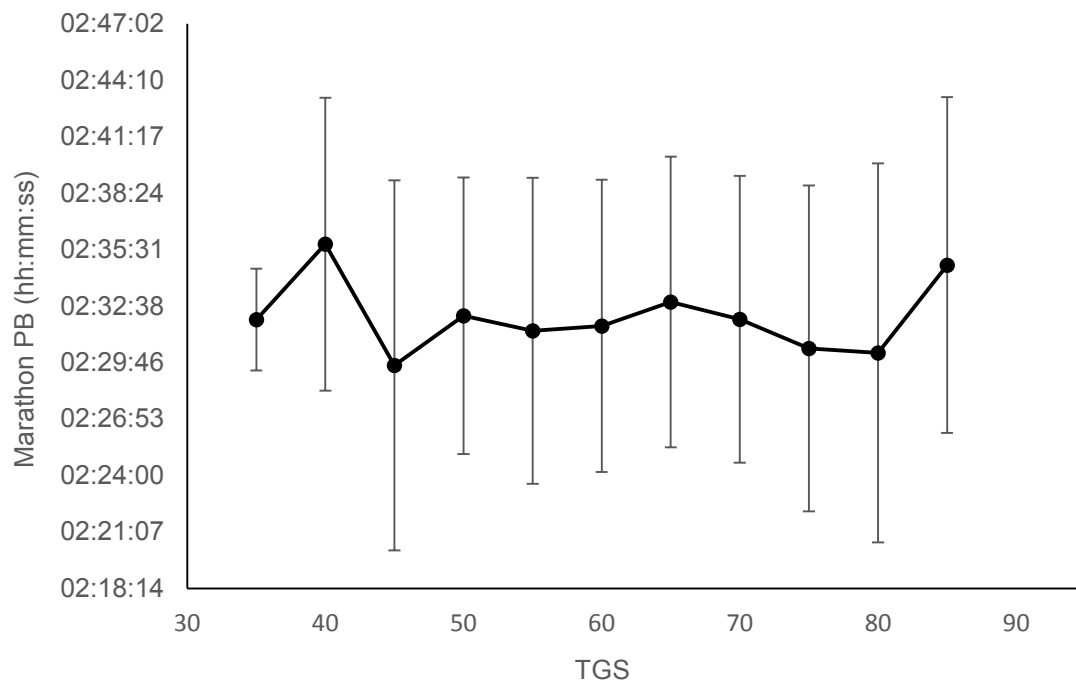
**Figure 4.6d:** TGS frequency distribution in the elite, sub-elite, top 10% and bottom 10% PB female runners.

## Marathon Performance

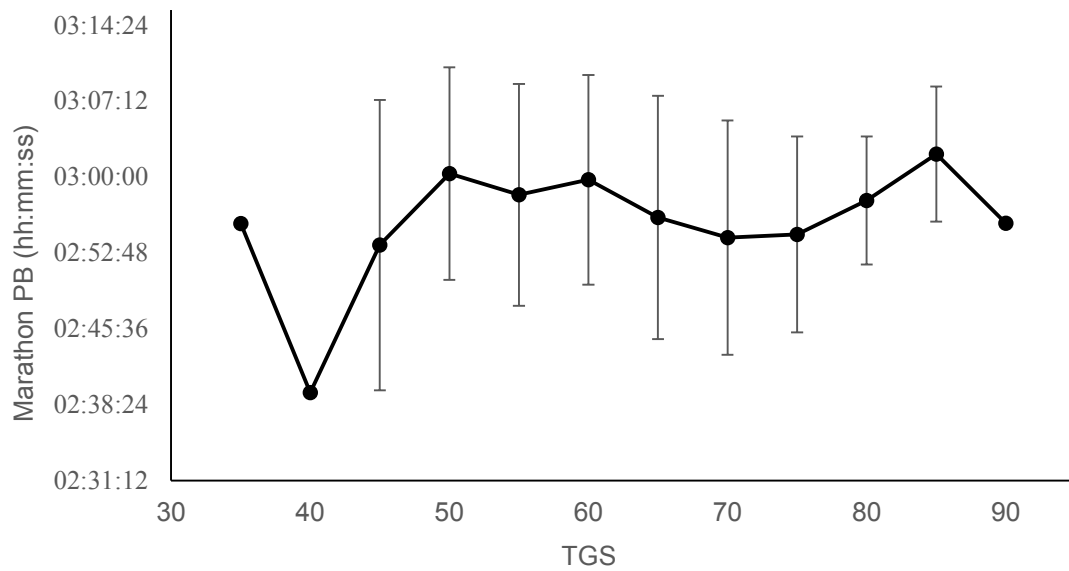
Personal best times were not associated with any gene variant individually for men ( $P \geq 0.086$ ), women ( $P \geq 0.183$ ; Table 4.4) or collectively as a TGS in men ( $P = 0.835$ ) or women ( $P = 0.257$ ). Additionally, TGS could not predict marathon PB for either males ( $\beta = -0.006$ ,  $P = 0.914$ ; Figure 4.7a) or females ( $\beta = -0.027$ ,  $P = 0.705$ ; Figure 4.7b) via linear regression.

**Table 4.4:** Mean personal best time (h:mm:ss) and associated genotype for all 10 investigated SNPS in both male and female runners. Uncorrected probability values are reported.

SNP	Genotype	PB (SD)		<i>P</i> - value	
		Males	Females		
<i>AXIN1</i> rs9921222	CC	2:31:33 (0:08:00)	2:57:19 (0:08:49)	0.781	0.581
	CT	2:32:04 (0:07:39)	2:56:09 (0:11:31)		
	TT	2:31:24 (0:07:30)	2:58:01 (0:11:20)		
<i>BDNF-AS</i> rs6265	GG	2:31:35 (0:08:01)	2:57:38 (0:10:30)	0.823	0.877
	GA	2:32:08 (0:07:07)	2:55:14 (0:11:24)		
	AA	2:32:09 (0:07:14)	2:55:38 (0:09:09)		
<i>COL1A1</i> rs1800012	AA	2:31:58 (0:07:55)	2:56:59 (0:10:30)	0.837	0.877
	AC	2:31:25 (0:07:30)	2:57:11 (0:11:43)		
	CC	2:31:39 (0:04:55)	2:54:50 (0:14:00)		
<i>COMT</i> rs4680	GG	2:30:55 (0:06:57)	2:57:31 (0:09:51)	0.086	0.856
	GA	2:31:37 (0:07:49)	2:56:35 (0:11:17)		
	AA	2:33:43 (0:07:42)	2:57:18 (0:10:24)		
<i>LRP5</i> rs3736228	CC	2:31:56 (0:07:33)	2:57:15 (0:10:13)	0.745	0.755
	CT	2:31:17 (0:08:16)	2:56:16 (0:11:44)		
	TT	2:32:54 (0:04:09)	2:53:50 (0:20:24)		
<i>P2RX7</i> rs3751143	AA	2:31:55 (0:07:47)	2:56:14 (0:10:51)	0.252	0.378
	AC	2:31:55 (0:07:11)	2:58:06 (0:10:18)		
	CC	2:27:49 (0:09:55)	2:59:53 (0:10:48)		
<i>TNFRSF11A</i> rs3018362	GG	2:32:28 (0:07:11)	2:57:49 (0:10:22)	0.331	0.568
	GA	2:31:31 (0:08:02)	2:56:07 (0:11:26)		
	AA	2:30:35 (0:07:53)	2:56:50 (0:09:11)		
<i>TNFRSF11B</i> rs4355801	AA	2:31:18 (0:08:24)	2:57:42 (0:10:13)	0.744	0.639
	AG	2:32:04 (0:07:10)	2:57:01 (0:11:07)		
	GG	2:31:50 (0:07:51)	2:55:37 (0:10:21)		
<i>VDR</i> rs2228570	CC	2:31:14 (0:07:07)	2:57:30 (0:10:22)	0.396	0.819
	CT	2:32:26 (0:08:03)	2:56:30 (0:11:10)		
	TT	2:31:21 (0:07:55)	2:57:15 (0:09:57)		
<i>WNT16</i> rs3801387	AA	2:32:12 (0:07:04)	2:58:20 (0:10:54)	0.621	0.183
	AG	2:31:23 (0:08:04)	2:55:39 (0:10:34)		
	GG	2:32:16 (0:09:29)	2:54:52 (0:07:29)		



**Figure 4.7a:** Mean marathon PB for each potential TGS of the male runners.



**Figure 4.7b:** Mean marathon PB for each potential TGS of the female runners.



#### 4.4 Discussion

This is the first time the influence of BMD-associated genetic variants have been investigated in a large cohort of high-level endurance runners in comparison to non-athletes and in relation to marathon personal best time. Elite runners possessed a lower frequency of the “risk” (associated with lower BMD) AA and GG genotypes in the *WNT16* rs3801387 and *COMT* rs4680 variants, respectively, in comparison to non-athletes and sub-elites. The “risk” C allele was more frequent in the runners than the control group for the *P2RX7* rs3751143 SNP.

Although elite runners possessed a higher frequency of the *WNT16* rs3801387 “risk” A allele in comparison to non-athletes in the dominant analysis model, they possessed a lower frequency of the AA genotype (44.9%) than non-athletes (49.4%) and sub-elite runners (55.5%). *WNT16* is as an important member of the Wnt signalling pathway known to influence BMD, cortical bone thickness and osteoporotic fracture risk (Zheng et al., 2012). The *WNT16* rs3801387 A allele has consistently exhibited associations with lower BMD via GWAS and candidate gene association study in adults and children (Koller et al., 2013; Estrada et al., 2012; Warrington et al., 2015). These findings therefore, may suggest that not possessing the AA genotype is beneficial for reaching elite status through protection against stress fracture injury and potential training interruption but does not influence initial capacity to reach high-level marathon performance.

*WNT16* and *AXIN1* are both Wnt signalling loci, which is known to influence the mechanosensitivity of the skeleton (Hens et al., 2005; Johnson and Kamel, 2007). Interestingly, both the *WNT16* rs3801387 and *AXIN1* rs9921222 variants have been reported to have nominal associations with higher BMD via gene-physical activity interactions previously (Mitchell et al., 2016), although no differences in allele

frequency for the *AXIN1* rs9921222 variant were observed after multiple testing correction. Mechanical loading leads to an increase in Wnt production by osteocytes, which activates the signalling pathway (Klein-Nulend et al., 2012). The concept of a gene-physical activity interaction has had limited investigation thus far, although previous results have suggested certain genotypes may be more sensitive than others to mechanical loading. For example, the *LRP5* rs2306862 and rs3736228 TT genotypes were associated with lower BMD in men with higher physical activity level but higher BMD in those with a lower physical activity level. However, the *LRP5* rs3736228 SNP did not present any significant findings in this chapter. This may be due to the effects of other proteins within the Wnt signalling pathway that may be more sensitive to mechanical loading in general or particular types of mechanical loading. SOST and Dickkopf (DKK) bind to LRP5/LRP6 which inhibits the Wnt signalling pathway by preventing Wnt ligand binding, reducing bone formation (Kawano and Kypta, 2003; Williams and Insogna, 2009). Deletion of *SOST* has been associated with insensitivity in unloading whilst *SOST* expression is also varied depending upon the nature of the loading conditions (Lin et al., 2009). Therefore, the influence of loading may mediate other components of the Wnt signalling pathway (such as SOST) and consequently differences in *LRP5* genotype may not be as important for bone phenotypes within this mechanism. Additionally, young adults were the investigated cohort in the Mitchell et al. (2016) study and childhood appears to be a key point in time for bone accrual and age-specific effects for particular loci on BMD has been reported (Medina-Gomez et al., 2012). This age-associated influence and potential gene-physical activity interaction emphasises the difficulty in making appropriate or direct comparisons/conclusions and may explain why contradictory results, such as finding no association with the *LRP5* rs3736228 variant in this investigation, are present.

Elite runners possessed a much lower frequency of the “risk” GG *COMT* rs4680 genotype in comparison to non-athletes (16.6% vs 24.0%) and the sub-elite athletes possessed the highest frequency of the GG genotype (24.6%). These findings are similar to the *WNT16* rs3801387 variant, suggesting that elite athletes may be genetically predisposed to be at less risk of lower BMD by possessing at least one “protective” A allele, although no differences in allele frequency were observed. GA genotypes were more prominent in the runner group in comparison to non-athletes (52.8% vs 47.2%) and an even larger representation was present in the elite runners (56.3%) when divided into subgroups. *COMT* catalyses the methylation of catechol oestrogens to methoxy oestrogens (inactive metabolites) and thus, lower *COMT* enzyme activity results in greater 16-hydroxy-oestradiol, which retains oestrogenic activity and enhances BMD (Lorentzon et al., 2007). Consequently, both higher oestradiol serum levels (Eriksson et al., 2004) and higher BMD (Eriksson et al., 2005) have been reported in lower enzyme activity AA genotypes. Conflicting results regarding the more beneficial *COMT* rs4680 genotype for BMD, however, have been observed (Gonçalves et al., 2015) which, may be due to the effect of mechanical loading.

The *COMT* rs4680 SNP has been shown to influence the association between physical activity and BMD, suggesting that certain genotypes may be particularly important for BMD in individuals with low physical activity levels. Higher total BMD has been observed in individuals completing greater levels of physical activity compared to those undertaking lower activity for GA and AA (lower enzyme activity) but not GG (higher enzyme activity) genotypes (Lorentzon et al., 2007). A *COMT* genotype interaction, thus, may be present and the potential regulation of the BMD response to mechanical loading may be due to the involvement of oestrogen receptors as facilitators in a number of key pathways by which mechanical strain stimulates bone formation (Galea

et al., 2013). This notion, due to the substantial amount of mechanical loading marathon runners undertake, may explain why the GA genotype was overrepresented in the elite group. Therefore, possessing the GA genotype may be advantageous for BMD via a mechanical loading interaction, allowing runners to complete the necessary training volumes with a smaller chance of stress fracture occurrence to reach elite status.

Surprisingly, the “risk” C allele of the loss of function *P2RX7* rs3751143 was more frequent in the runners than non-athletes, with non-athletes possessing a higher frequency (>6%) of the advantageous AA genotype. Functional expression of purinergic receptor P2X 7 primarily regulates configuration of osteoclasts (Agrawal et al., 2010), as well as augmenting bone formation via a cell-autonomous role that leads to stimulation of mineralisation (Panupinthu et al., 2008) and thus, may be important for increasing BMD. Previous studies in military recruits and elite athletes have also found the C allele to be associated with stress fracture incidence (Varley et al., 2016). A lower frequency of the C allele in the runners than non-athletes and the subsequent protection from large volumes of mechanical loading, would therefore, be expected. The *P2RX7* gene codes for a trimeric ligand-gated cation channel, which, when activated by ATP, results in the release of pro-inflammatory mediators and cell proliferation. Consequently, some *P2RX7* variants have been implicated in a number of human health or related conditions, such as Tuberculosis and Rheumatoid arthritis as well as osteoporosis (Sluyter and Stokes, 2011). The *P2RX7* rs3751143 CC loss of function genotype has been associated with reduced risk of ischemic stroke and P2X7 deficient mice have been reported to be protected from thrombosis in vivo (Gidlöf et al., 2012). Therefore, although the elite runners may possess the genotype associated with lower BMD, it could be protective or advantageous in other important determinants

for endurance success, such as cardiovascular factors, albeit speculative at this moment in time.

A higher prevalence of the *VDR* rs2228570 CC genotype in runners than the non-athlete group (39.6 vs 35.4%) was observed before multiple testing correction, which presents an interesting finding given previous studies on this variant. The CC genotype has been reported to be more frequent in track and field athletes than controls and associated with higher BMD (Nakamura et al., 2002b). This notion was further reinforced in a similar investigation in 44 Japanese track and field athletes, where higher bone volume was expressed in those with the CC genotype, but not in those with the CT genotype (Nakamura et al., 2002a). Interestingly, within the control group only, the CC genotype was not associated with the highest BMD, thus, suggesting that individuals with the CC genotype may be more responsive to mechanical loading, resulting in greater BMD, when physical activity is prominent. Together, these aforementioned findings, known biological function of the associated protein and the tendency observed in this chapter could suggest a potential benefit for enhancing BMD and reducing injury risk for high-level endurance runners possessing the CC genotype. Future research capturing the athlete-associated BMD data could provide evidence that the CC genotype aids in achieving high-level endurance runner status through enhanced BMD (via a potential gene-physical activity interaction) and subsequent protection against stress fracture injury. No association was observed when comparing elite runners against non-athletes or when analysing an association with PB in this investigation, which may suggest the CC genotype provides protection to reach high-level status but does not influence performance once this level has been achieved.

A difference in *TNFRSF11A* rs3018362 allele frequency between runners and non-athletes was only observed before multiple testing correction and no differences in genotype or allele frequencies were discovered for the other four investigated variants

(*BDNF-AS* rs6265, *COL1A1* rs1800012, *LRP5* rs3736228 and *TNFRSF11B* rs43558010). Personal best times were also not associated with any individual genotype in both males and females (Table 4.4) or collectively as part of TGS. Additionally, TGS could not predict PB via regression analysis. These findings suggest that the investigated genetic variants do not influence marathon PB singularly or collectively as part of a TGS. Successful endurance performance is multi-factorial and genes influencing other important phenotypes, such as  $\text{VO}_2$  max or lactate threshold (Sleivert and Rowlands, 1996; Jones and Carter, 2000), are also likely to be influential in determining performance.

Mean TGS was investigated between the runners and non-athletes as well as between sub-groups as a whole and by sex (Table 4.3). No differences were observed in any comparison although a tendency (before multiple testing correction) highlighting that the elite group possessed a higher mean TGS than the sub-elite runners was observed. It was also interesting to note that a tendency for a higher TGS frequency distribution in the top 10% compared to the bottom 10% (according to PB) in the female runners via ROC analysis was observed but no such tendency was apparent in the equivalent male comparison. Grealy et al. (2015) found comparable findings in the top 10% vs the bottom 10% performers in Ironman Championship male and female triathletes when investigating variants associated with endurance performance. A shift towards a higher TGS distribution was apparent alongside a higher TGS mean (+7) in the fastest 10% in comparison with the bottom 10% finishers. Similarly, TGS was not significantly different between the groups and not associated with performance time. It is acknowledged that the 10% vs 10% analysis consists of a small sample size but the top 10% female group comprises runners who all have a PB of < 2 h 41 min for the marathon, which is notably faster than the current 2 h 45 min Olympic qualifying time. This could suggest having an advantageous genetic profile in relation to BMD could

provide a small contribution for female endurance runners to achieve elite status on top of possessing other advantageous genotypes for VO<sub>2</sub> max, lactate threshold or running economy. Female athletes are more prone to stress fractures (Milner et al., 2006) due to lower BMD through potential complications of RED-S via irregular menses and/or energy deprivation (Loucks, 2007). Possessing a more advantageous genetic profile and consequently higher BMD, therefore, may provide greater protection from bone injury in female runners completing rigorous training volume/intensity. TGS provides an interesting model to analyse potential associations with athletic status or performance in a polygenic rather than singular nature, particularly due to the difficulty of calculating the effect size of single or multiple polymorphisms. However, this is also the primary limitation of the model. The assumption of an additive effect between genotypes and that all variants are given the same weight in the total score, suggests each variant explains the same proportion of variance within the phenotype (Eynon et al., 2011) which is extremely unlikely. Further understanding of the genetic influence of specific SNPs will allow more accurate allocation of variance in the future to enhance the model.

The findings regarding genotype/allele frequency differences and influence on PB emphasise the complex and multifaceted nature of successful marathon performance, which is comprised of many more components including volume of training, VO<sub>2</sub> max and economy of movement. Similarly, BMD is also a multifactorial phenotype influenced by hormones and diet as well as physical activity. These complex traits and their individual components are undoubtedly polygenic, influenced by a large number of genes and subsequent gene-gene and gene-environment interactions and thus, require further investigation to better our understanding of the genetic contribution. Over 66 loci have been associated with BMD via GWAS and many variants have not been investigated further or explored in other populations (Hsu and Kiel, 2012) whilst

no common variant profile has been attributed to world-class endurance athletes (Rankinen et al., 2016). Although there is a reasonable volume of evidence highlighting the association between particular SNPs and BMD (Hsu and Kiel, 2012) as well as BMD and stress fracture injury (Bennell et al., 1996), this approach linking SNP to BMD to stress fracture injury and subsequently performance is somewhat speculative due to the complex and multi-factorial nature of BMD, stress fracture injury and performance as individual concepts. This hypothetical approach also aligns with the findings from the thesis as this complex hypothesis is not portrayed or followed through as a finding in the data. Consequently, additional research to establish the initial genetic associations with BMD as well as low BMD and stress fracture injury risk is needed so this approach can be further developed.

This study is the first to investigate any potential association of bone mineral density-related genes and marathon performance in high-level endurance runners. The *COMT* rs4680 and *WNT16* rs3801387 variants may aid in achieving elite marathon runner status but do not influence performance once high-level status has been attained. Surprisingly, the risk C allele of the *P2RX7* rs3751143 variant is more frequent in high-level endurance runners than non-athletes, although the mechanisms underlying why this may be the case are not yet understood. Additionally, the other investigated variants singularly, or collectively as part of a TGS, did not influence athlete status or marathon PB, although an advantageous polygenic predisposition may be of a slight benefit for female elite marathon runners at Olympic qualifying level. Therefore, these results suggest that elite runners may benefit from genetic resistance to bone injury in the *COMT* rs4680 and *WNT16* rs3801387 variants, and a resulting ability to sustain large training volumes and achieve successful marathon performance. Further research incorporating BMD data and injury history alongside the associated genotype information is required to provide more conclusive evidence.



## **Chapter 5:**

# **Genetic associations with bone mineral density in high-level endurance runners and non-athletes**

## 5.1 Introduction

Bone mineral density (BMD) is considered to be the primary predictor of osteoporotic fracture (Cranney et al., 2007) and is also important in the elite sporting environment, where athletes possessing lower BMD are proposed to be at greater risk of stress fracture injury (Myburgh et al., 1990; Bennell et al., 1999). Several factors such as diet, hormones, and physical activity are known to influence BMD, contributing to the large variability that exists within the phenotype (Pluijm et al., 2001; Krall and Dawson-Hughes, 1993).

A genetic influence on BMD also exists, with heritability reported as approximately 50-80% (Ralston and Uitterlinden, 2010). Genome-wide association studies (GWAS) and case-control designs have revealed many loci associated with variation in BMD. A number of the candidate genes identified at these loci, however, have no known associated biological function or have yet to be replicated in subsequent investigations to confirm associations with BMD (Hsu and Kiel, 2012). Furthermore, few investigations have considered gene-environment interactions - in particular, whether specific genes may be sensitive to mechanical loading from physical activity and the outcome of such an interaction for BMD and potential injury risk.

Mitchell et al. (2016) investigated the genomic influence on BMD and the relationship with physical activity using SNPs that had been associated with BMD via GWAS (Estrada et al., 2012). Analysis revealed nominal interactions with physical activity at the lumbar spine with variants such as *WNT16* rs3801387, *AXIN1* rs9921222, *SOST* rs4792909 and *STARD3NL* rs6959212 in children. Other variants such as *VDR* FokI rs2228570 have also been suggested to exhibit gene-physical activity interactions, with the TT (ff) genotype associated with higher BMD in active postmenopausal women (Gentil et al., 2009). When investigating the same SNP (*VDR* FokI rs2228570)

in athletes, however, significantly higher total BMD was observed in the CC and CT but not TT genotypes compared to 80 non-athlete controls. Interestingly, in a subgroup of swimmers from within this athlete population, a lower total BMD was observed in the CC genotype when compared to non-athlete controls (Nakamura et al., 2002a). Together, these findings suggest that individuals with the CC genotype may be more responsive to mechanical loading, resulting in greater BMD when that mechanical loading is prominent and therefore, contradicting the findings reported in postmenopausal women (Gentil et al., 2009). Physical activity levels and other factors, such as the effect of cessation of menopause, however, may be influencing the findings from the study investigating postmenopausal women so a direct comparison between the two populations cannot be completed. Only one other study to date has investigated gene-physical activity interactions and the outcome for bone phenotypes in athletic populations according to the authors' knowledge. Variants such as *SOST* rs1877632, *TNFRSF11A* rs9594738, *TNFSF11* rs1021188 and *TNFRSF11B* rs9594759 were associated with bone phenotypes before completion of a 12-week training programme in academy football players but no genotype by time interactions were observed (Varley et al., 2018). Consequently, proposed genetic associations with bone phenotypes were observed but no genetic interactions with mechanical loading were apparent and therefore, conflict previous investigations (Nakamura et al., 2002b; Nakamura et al., 2002a).

Overall, only a small number of BMD-associated variants have been explored in athletic populations or in relation to gene-physical activity interactions and thus, the potential influence on BMD in athletic populations is not yet fully understood. Individuals who complete higher levels of weight-bearing physical activity tend to possess higher BMD but it is not yet known which specific genetic variants may contribute to this increased BMD and whether this is via a potential gene-physical

interaction, or if those individuals predisposed to having a higher BMD self-select weight-bearing physical activities and sports in which to partake. Understanding of the genetic association with BMD could have substantial future implications for athlete health, status and performance. For athletes specifically, a manipulation of training load or practices would certainly be useful for athletes who possess a disadvantageous genetic disposition.

Athletic populations present the most logical participants to investigate potential gene-physical activity interactions due to the difficulty in assessing physical activity volume as discussed in Chapter 1 of this thesis. Endurance runners, in particular, represent a homogenous group that experience high volumes of mechanical loading at certain sites (e.g. tibia) as well as areas of less loading such as the lumbar spine. Thus, this group presents an ideal population in which to investigate the potential gene-physical activity interactions for BMD.

The purpose of this study, therefore, (1) was to investigate any association of the ten BMD-associated variants described in Chapter 1 with total-body BMD (TBMD), leg BMD (LBMD), lumbar spine BMD (LSBMD), total-body T-score and total-body Z-score in high-level endurance runners and non-athletes and (2) explore whether a genotype-dependent influence on the adaptations of bone phenotypes to long-term mechanical loading was present in the runners.

## **5.2 Method**

The participants and protocols used in Chapter 5 have already been described in detail in Chapter 2, thus, only a brief description of these methods is detailed below.

### *Participant characteristics*

Participants consisted of 103 high-level Caucasian runners (45 males, 58 females) and 112 ethnically matched non-athletes (52 males, 60 females) from the cohort as described in Table 2.1 of Chapter 2.

### *Protocol*

All runners completed a questionnaire detailing ethnic ancestry, as well as performance, injury, and sporting history (Appendix 1). Non-athletes completed a questionnaire detailing ethnicity, to establish matched ethnic ancestry, general health and physical activity level to ensure no history of high-level sporting competition (Appendix 2). All participants completed a whole-body DXA scan to gather BMD ( $\text{g/cm}^2$ ) data with whole-body and segmental analysis utilised to obtain TBMD, LBMD, LSBMD, T-score and Z-score. All participants provided a whole-blood, saliva or buccal swab sample, which was subsequently extracted and analysed to obtain genotype data for the ten investigated SNPs (*AXIN1* 9921222, *BDNF-AS* rs6265, *COL1A1* rs1800012, *COMT* rs4680, *LRP5* rs3736228, *P2RX7* rs3751143, *TNFRSF11A* rs3018362, *TNFRSF11B* rs4355801, *VDR* rs2228570, *WNT16* rs3801387) as described in Chapter 2.

### *Statistical analysis*

Using both an additive and dominant analysis model, multiple analysis of variance (MANOVA) was implemented to determine associations between any of the ten variants (individually and then collectively as a TGS) with TBMD, LBMD, LSBMD, T-score or Z-score in the total cohort (runners plus non-athletes) as well as within runners and non-athletes independently. The MANOVA model was also used to assess potential differences in any bone parameter between the runners and non-athletes with the same genotype (i.e. a genotype-cohort interaction). Significant associations from

any MANOVA model (i.e. main effect of genotype on any bone phenotype or a genotype-cohort interaction effect on any bone phenotype) were then subjected to sex-dependent pairwise statistical analyses among each of the two or three genotype groups. Consequently, independent t-tests (corrected for multiple testing via Benjamini-Hochberg) were conducted to analyse genotype-dependent differences in any bone parameter following a significant main effect of genotype on the total cohort, runners or non-athletes from the initial MANOVA model. Similarly, following a significant genotype-cohort interaction on a bone phenotype, independent t-tests were used to analyse cohort-dependent differences in these bone phenotypes across the same genotypes. Alpha was set at 0.05 for the main effect of genotype and significant genotype-cohort interaction but tendencies such that  $P > 0.05$  but  $< 0.15$  (Danilovic et al., 2007; Fischer et al., 2004) were reported for the subsequent pairwise analyses as and data are reported as mean (SD).

### 5.3 Results

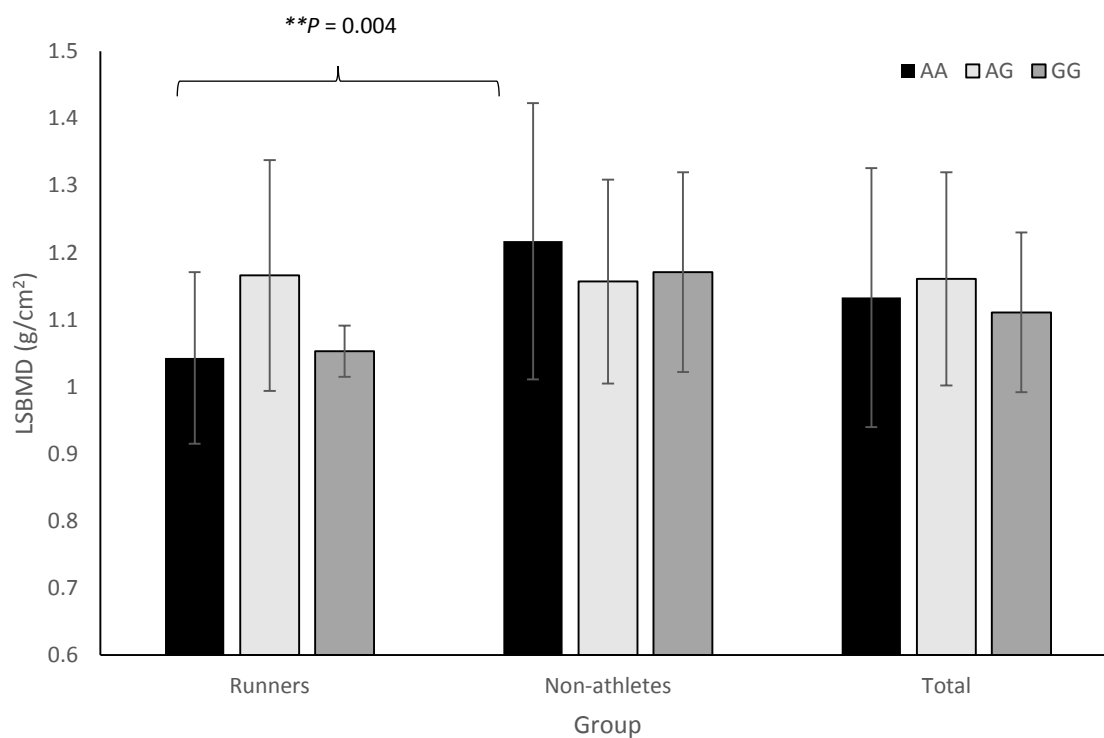
#### *Genotype-cohort interaction*

Genotype-cohort interaction analyses (Tables 5.1 and 5.2) are provided within the appendices (Appendix 3 and 4, respectively). A genotype-cohort interaction was observed for the *WNT16* rs3801387 variant in males and *BDNF-AS* r6265 variant in females. Specifically, a genotype-cohort interaction was present for the LBMD, LSBMD and Z-score but not TBMD or T-score for the *WNT16* rs3801387 variant in the additive model analysis ( $P = 0.032$ ;  $P = 0.042$ ;  $P = 0.045$ ;  $P = 0.057$ ;  $P = 0.051$ ; Table 5.1). In the *BDNF-AS* rs6265 variant, the genotype-cohort interaction was observed for LSBMD in the dominant analysis model only ( $P = 0.037$ ).

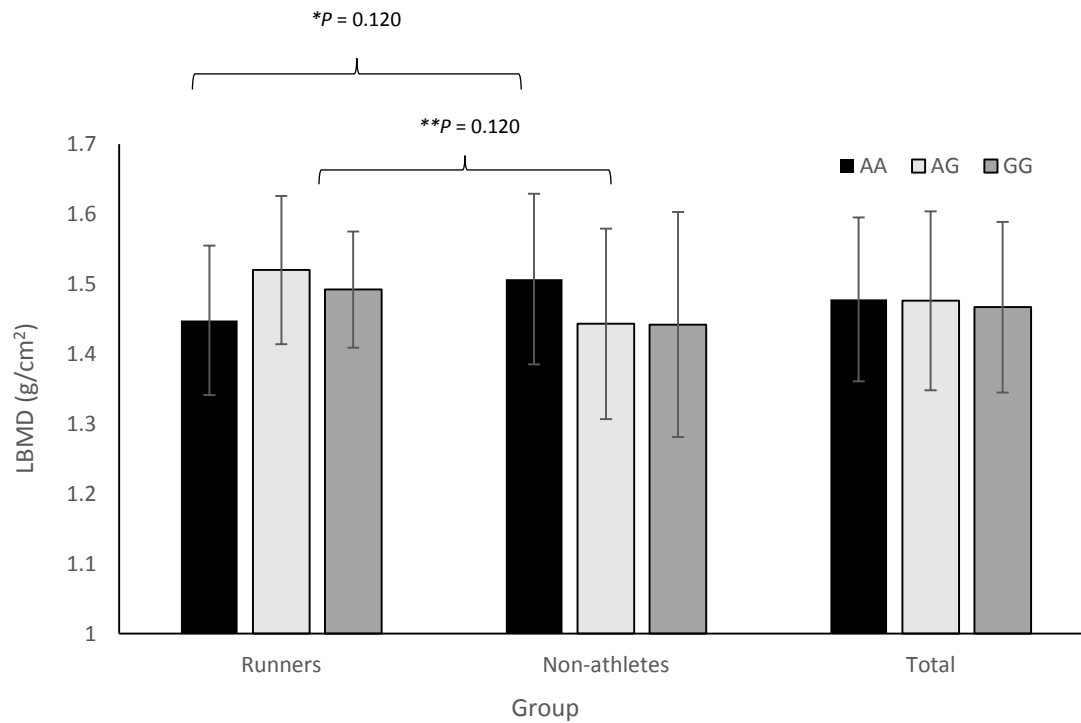
Within the *WNT16* rs3801387 interaction, runners with AA genotype possessed lower (~17%) LSBMD and Z-score ( $P = 0.004$ ; Figure 5.1; 0.62 vs 1.35;  $P = 0.036$ ; Table

5.1) and a tendency to possess lower LBMD than non-athletes with AA genotype ( $P \geq 0.120$ ; Figure 5.2). Additionally, a tendency for runners with AG genotype to possess higher LBMD than non-athletes with AG genotype was observed ( $P = 0.120$ ; Figure 5.2).

In the *BDNF-AS* rs6265 interaction, runners with the GG genotype possessed ~9% lower LSBMD than non-athletes with the GG genotype ( $P = 0.022$ ; Figure 5.3). No differences in LSBMD were observed between runners who carried the A allele in comparison to non-athletes who were A allele carriers ( $P = 0.416$ ; Figure 5.3). No other genotype-cohort interactions with any bone phenotypes were observed for either males or females for any other variant ( $P \geq 0.057$ ; Table 5.1; Table 5.2).

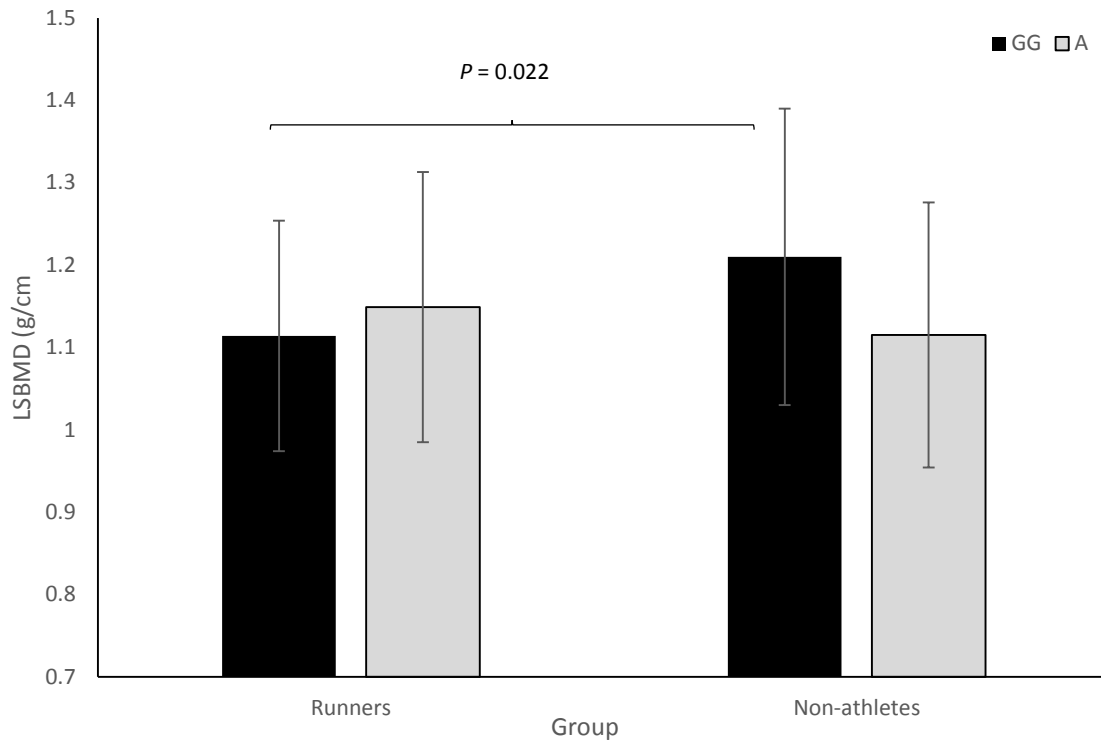


**Figure 5.1:** LSBMD in *WNT16* rs3801387 AA, AG and GG genotypes in male runners, male non-athletes and the male total cohort (runners + non-athletes). Higher LSBMD in AA genotype non-athletes than AA genotype runners ( $P = 0.004$ ). Error bars denote standard deviation.



**Figure 5.2:** LBMD in *WNT16* rs3801387 AA, AG and GG genotypes in male runners, male non-athletes and the male total cohort (runners + non-athletes). Tendency for lower LBMD in AA genotype runners than AA genotype non-athletes ( $*P = 0.120$ ). Tendency for higher LBMD in AG genotype runners than AG genotype non-athletes ( $**P = 0.120$ ). Error bars denote standard deviation.





**Figure 5.3:** LSBMD in *BDNF*-AS rs6265 GG and A allele carriers genotypes in male runners and male non-athletes. Lower LSBMD in GG genotype runners than GG genotype non-athletes ( $P = 0.022$ ). Error bars denote standard deviation.

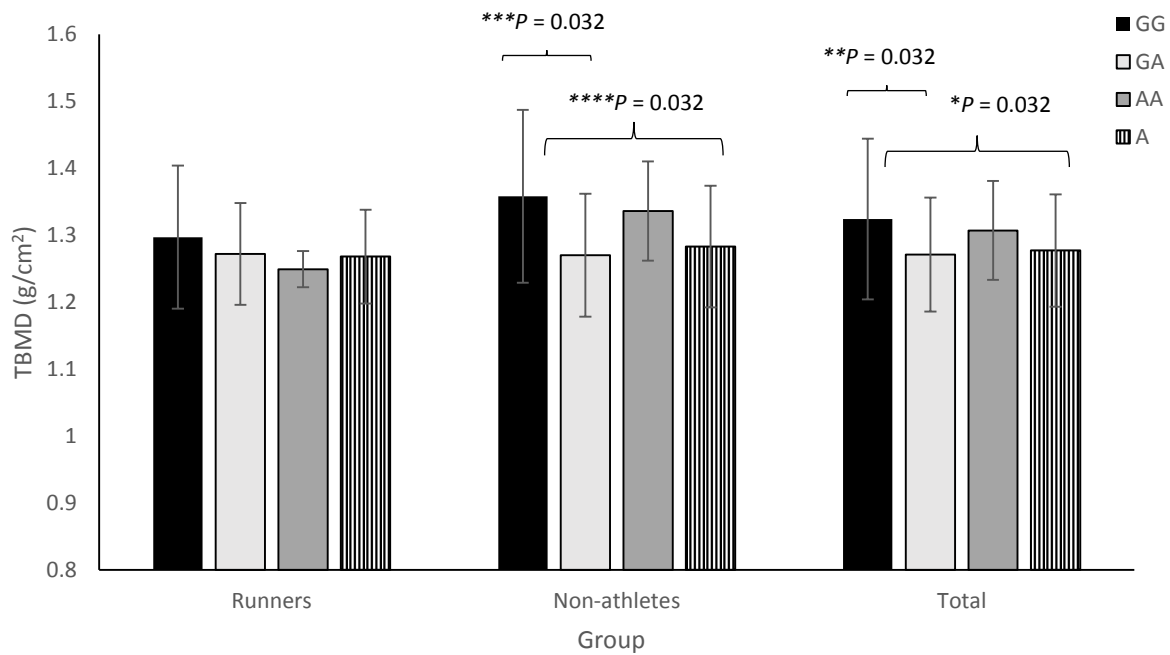
#### Genetic association

##### Males

A main effect of *TNFRSF11A* rs3018362 genotype on TBMD was observed in the males for both the additive and dominant analysis models ( $P \leq 0.043$ ; Table 5.1). Specifically in the total cohort, those with the *TNFRSF11A* rs3018362 GG genotype possessed ~4% higher TBMD than GA genotypes ( $P = 0.032$ ; Figure 5.4). In non-athletes, GG genotypes possessed higher TBMD than GA genotypes ( $P = 0.032$ ; Figure 5.3). No further genotype-dependent differences in TBMD were observed within non-athletes ( $P \geq 0.160$ ) and no main effect of *TNFRSF11A* rs3018362 genotype on TBMD was present in the runners ( $P = 0.619$ ; Figure 5.4).

In the dominant analysis model, those who were GG genotypes possessed higher TBMD (~4%) than the “risk” A allele carriers in the male total cohort ( $P = 0.032$ ; Figure

5.4). In the non-athlete analysis, similarly, those who were GG genotypes possessed higher TBMD than those who carried the A allele ( $P = 0.032$ ). No main effect of *TNFRSF11A* rs3018362 genotype on TBMD was apparent within the runners in the dominant analysis model ( $P = 0.362$ ).



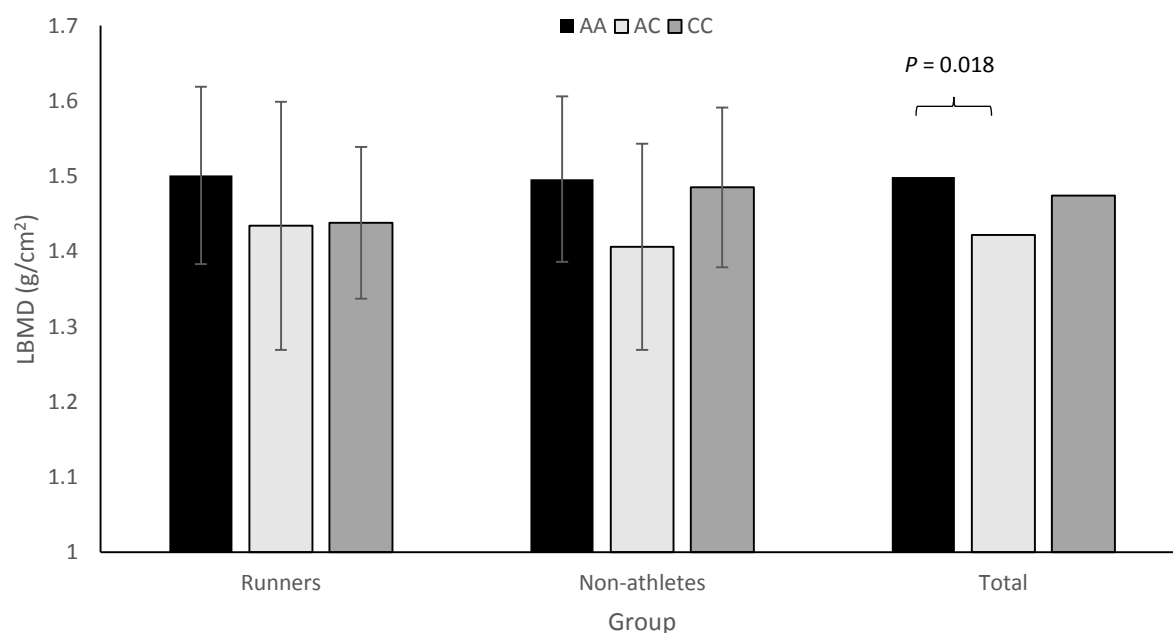
**Figure 5.4:** TBMD in G allele carriers, GG, GA and AA genotypes of the *TNFRSF11A* rs3018362 variant in the male runners, male non-athletes and total male cohort (runners + non-athletes). Higher TBMD in GG genotypes than A allele carriers in the total cohort ( $*P = 0.032$ ). Higher TBMD in GG than GA genotypes in the total cohort analysis ( $**P = 0.032$ ). Higher TBMD in GG than GA genotype non-athletes ( $***P = 0.032$ ). Higher TBMD in GG genotypes than A allele carriers in the non-athletes ( $****P = 0.032$ ). Error bars denote standard deviation.

A main effect of *COL1A1* rs1800012 genotype on LBMD and T-score was also observed in males ( $P = 0.021$ ;  $P = 0.033$ ; Table 5.1). Specifically in the total cohort, individuals with *COL1A1* rs1800012 AA genotype possessed ~5.5% higher LBMD than AC genotype counterparts ( $P = 0.018$ ; Figure 5.5). There was a tendency for *COL1A1* rs1800012 AA genotypes to possess a higher T-score than AC genotypes in both the male total cohort and non-athletes (both  $P = 0.051$ ). No other *COL1A1* rs1800012 genotype-dependent differences in LBMD or T-score were observed for the total cohort

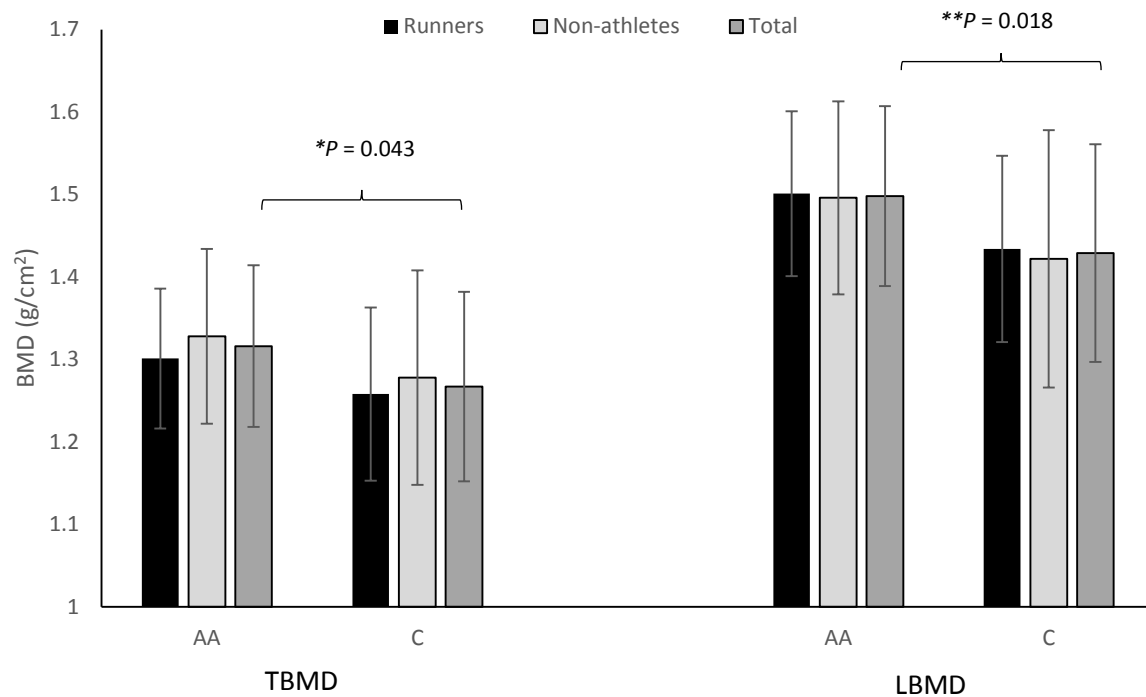
or non-athletes and no main effect of genotype was apparent in runners ( $P \geq 0.190$ ; Figure 5.5).

In the dominant analysis model, a main effect of *COL1A1* rs1800012 genotype in the total cohort was observed, with AA genotypes possessing higher TBMD, LBMD, T-score and Z-score than C allele carriers ( $P = 0.043$ ;  $P = 0.018$ ;  $P = 0.022$ ;  $P = 0.039$ ; Figure 5.6). No main effect of genotype was apparent in runners or non-athletes ( $P \geq 0.070$ ).

No other SNPS, individually or collectively as part of a TGS, were associated with any of the bone phenotypes in men ( $P \geq 0.168$ ).



**Figure 5.5:** LBMD in AA, AC and CC *COL1A1* rs1800012 genotypes in the male runners, male non-athletes and total male cohort. Higher LBMD in AA than AC genotypes in the total cohort ( $P = 0.018$ ). Error bars denote standard deviation.



**Figure 5.6:** TBMD and LBMD in *COL1A1* rs1800012 AA genotypes and C allele carriers in the male runners, male non-athletes and total male cohort. Higher TBMD and LBMD in AA genotypes than C allele carriers in the total cohort (\* $P = 0.043$ ; \*\* $P = 0.018$ ).

### Females

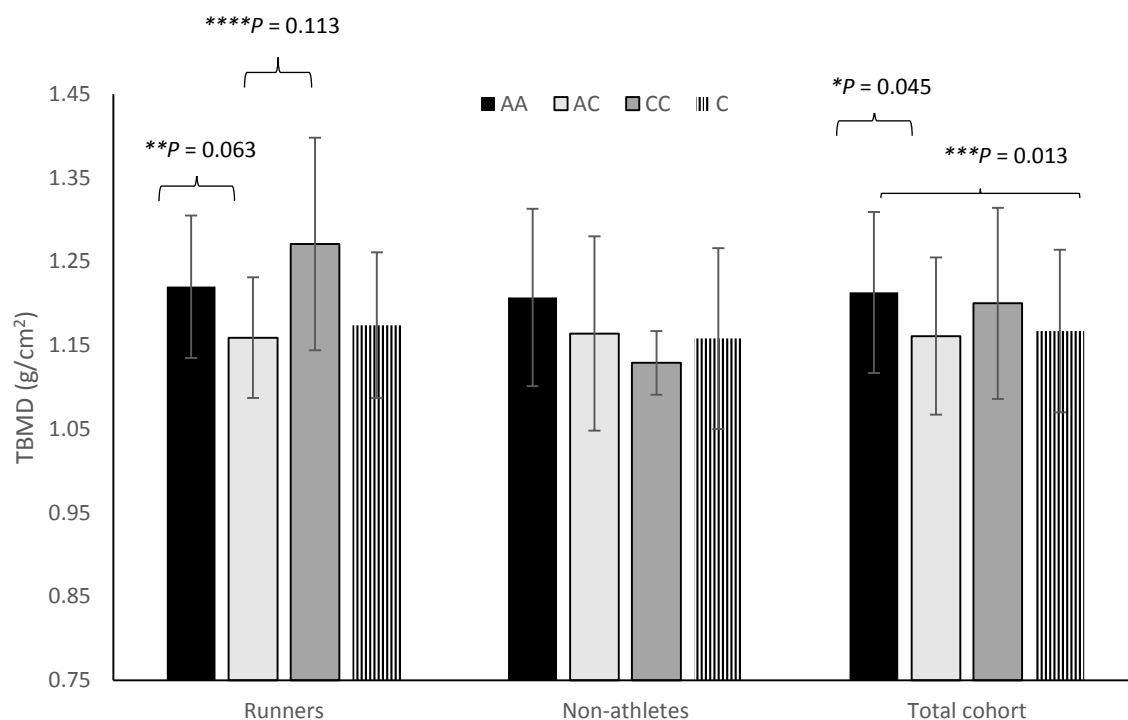
A main effect of *P2RX7* rs3751143 genotype on TBMD, LBMD, T-score and Z-score for the total cohort was observed in the female additive model ( $P = 0.030$ ;  $P = 0.048$ ;  $P = 0.030$ ;  $P = 0.022$ ; Table 5.2). AA genotypes possessed higher TBMD and LBMD than AC genotypes ( $P = 0.045$ , Figure 5.7;  $P = 0.036$ , Figure 5.8) and AA genotypes also exhibited a higher T-score and Z-score than AC genotypes ( $P = 0.042$ ;  $P = 0.028$ ).

A main effect of *P2RX7* rs3751143 genotype on TBMD, T-score and Z-score also existed within the runners ( $P \leq 0.043$ ). Specifically, a tendency for AA genotypes to possess higher TBMD and T-score than AC genotypes was observed ( $P = 0.063$ ;  $P = 0.061$ ), whilst a tendency for AC genotypes to possess lower TBMD and T-score than CC genotypes was also apparent ( $P = 0.113$ ;  $P = 0.130$ ; Figure 5.7). Runners with AA genotype possessed a higher Z-score than AC genotypes ( $P = 0.037$ ). No further

genotype-dependent differences for TBMD, T-score or Z-score were present within runners ( $P \geq 0.122$ ) and no main effect of *P2RX7* rs3751143 genotype on any bone phenotype was apparent in the non-athletes ( $P \geq 0.248$ ).

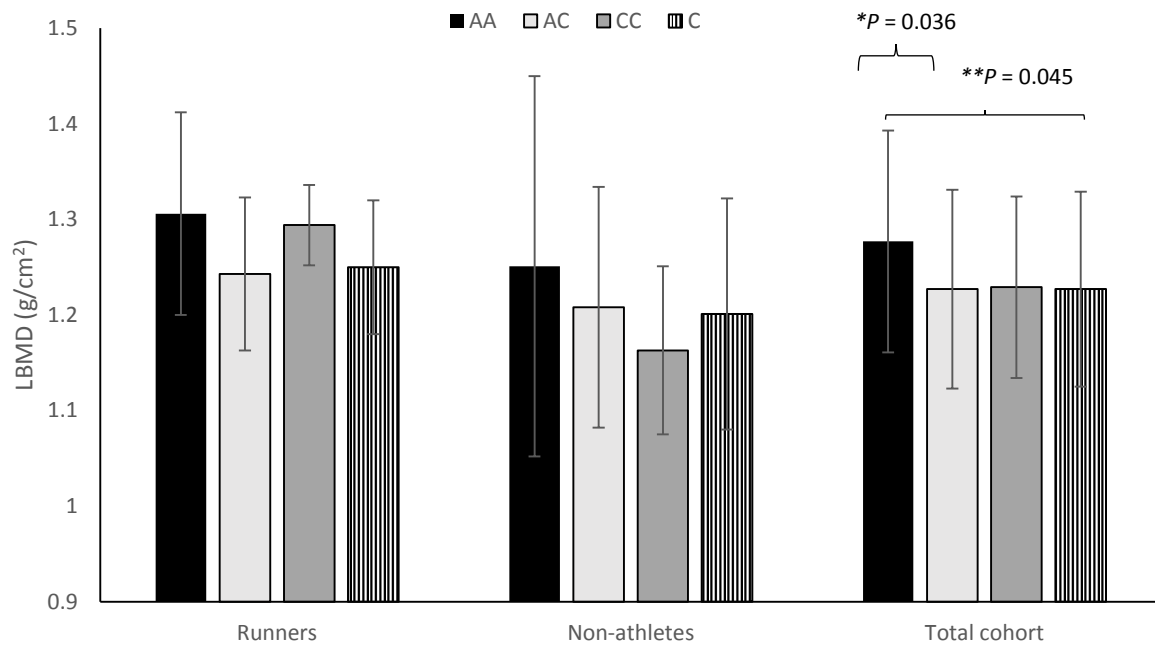
A main effect of *P2RX7* rs3751143 genotype on the total cohort TBMD in the dominant analysis model was also observed ( $P \leq 0.040$ ). Specifically, AA genotypes possessed 4% higher TBMD and LBMD, as well as a higher T-score and Z-score than those who were C allele carriers ( $P = 0.045$ ;  $P = 0.036$ ;  $P = 0.042$ ;  $P = 0.028$ ; Figure 5.7 and Figure 5.8). No main effect of *P2RX7* rs3751143 genotype on any bone phenotype was present in the runners or non-athletes ( $P \geq 0.061$ ).

No other SNPS, individually or collectively as part of a TGS, were associated with any of the bone phenotypes in women ( $P \geq 0.124$ ; Table 5.2).



**Figure 5.7:** TBMD in C allele carriers, AA, AC and CC genotypes of the *P2RX7* rs3751143 genotype in the female runners, female non-athletes and total female cohort (runners + non-athletes). Higher TBMD in AA than AC genotypes in the total cohort (runners + non-athletes). Higher TBMD in AA than AC genotypes in the total cohort (\* $P = 0.045$ ). Tendency for higher TBMD in AA than AC genotype runners (\*\* $P = 0.063$ ).

= 0.063). Higher TBMD in AA genotypes than C allele carriers in the whole cohort ( $***P = 0.013$ ). Tendency for higher TBMD in CC than AC genotype runners ( $****P = 0.113$ ). Error bars denote standard deviation.



**Figure 5.8:** LBMD in C allele carriers, AA, AC and CC genotypes of the *P2RX7* rs3751143 variant in the female runners, female non-athletes and total female cohort (runners + non-athletes). Higher LBMD in AA than AC genotypes in the total cohort ( $*P = 0.036$ ). Higher LBMD in AA genotypes than C allele carriers in the total female cohort ( $**P = 0.045$ ). Error bars denote standard deviation.

## 5.4 Discussion

The aim of this chapter was to investigate BMD-associated SNPs on TBMD, LBMD, LSBMD, T-score and Z-score in endurance runners and non-athletes as well as explore potential gene-mechanical loading interactions in these investigated SNPs by analysing gene-cohort interactions.

### *Genotype-cohort interactions*

Runners with *WNT16* rs3801387 AA genotype possessed lower LSBMD and Z-score and a tendency to possess lower LBMD than non-athletes with AA genotype, whilst a tendency for runners with AG genotype to possess higher LBMD than non-athletes

with AG genotype was also observed, highlighting an interaction between genotype and the two cohorts for the two anatomical sites. These findings could suggest that the G allele is important for BMD in male endurance runners via an adaptation to chronic high mechanical loading. The “risk” A allele has been associated with lower lumbar spine and femoral neck BMD via GWAS (Estrada et al., 2012) and candidate gene association studies (Hendrickx et al., 2014) in osteoporotic and non-athlete populations. In this chapter, no differences in BMD between *WNT16* rs3801387 genotypes in the non-athletes was observed and thus, contradicts these previous investigations. The difference in the findings could be due to the influence of mechanical loading completed by the running cohort, with the majority of studies having been conducted in non-athletic populations. Although a main effect of *WNT16* rs3801387 genotype was not present, and thus these differences were not significant, it is interesting to note that non-athletes with AA genotype possessed the highest LBMD and LSBMD in their group whereas AA genotype runners possessed the greater LBMD and LSBMD in their group. Additionally, a greater difference in BMD between GG and AA genotypes in both the runners and non-athletes was observed at the LBMD (where the site-specific bone adaptation to mechanical loading will occur in the endurance runners) than LSBMD, which may indicate the potential impact of a greater mechanically induced strain on bone adaption by possessing a G allele.

Wnt16 is predominantly expressed in osteoblasts and is an important member of the Wnt signalling pathway, activating both both canonical and non-canonical pathways and consequently, having substantial implications for cortical bone mass (Gori et al., 2015). Wnt16 is a key regulator of osteoblast-to-osteoclast communication and targeted disruption of Wnt16 in mice results in a 27% loss in bone size and 43-61% loss in bone strength (Zheng et al., 2012). *WNT16* knockout mice have exhibited substantial reductions (20-70%) in expression levels of canonical Wnt signalling

markers in the periosteum at 5 weeks of age in comparison to wild-type mice, suggesting that *Wnt16*, via Wnt signalling, moderates mechanical loading-induced periosteal bone formation and size (Wergedal et al., 2015). Additionally, the *WNT16* rs3801387 SNP has also been reported to interact with physical activity to demonstrate nominal associations with BMD in children and adolescents (Mitchell et al., 2016), providing further support for a gene-mechanical loading interaction on BMD. The findings from this chapter could therefore suggest that possessing a *WNT16* rs3801387 G allele is associated with higher LBMD in endurance runners compared to non-athletes due to the effect of mechanical loading on Wnt production via osteocytes, leading to activation of the Wnt signalling pathway, which subsequently impacts bone tissue via the canonical or non-canonical pathway. This interpretation, however, has to be taken with caution as no differences in the dominant model analysis or in the GG genotype between the runners and non-athletes were observed. Additionally, mechanically loaded mice have demonstrated no upregulation of *Wnt16* expression (Todd et al., 2015). Further functional and phenotype studies in human populations are therefore required to confirm this potential mechanism on BMD.

*BDNF-AS* rs6265 also exhibited a genotype-cohort interaction for LSBMD in the female analysis. Runners with the advantageous (higher BMD-associated) GG genotype possessed lower LSBMD than non-athletes who were GG genotype. Additionally, (although no main effect of genotype was present in the runners or non-athletes) runners with the A allele possessed higher LSBMD than the GG genotypes, whilst non-athletes with the GG genotype possessed higher LSBMD than the A allele carriers. *BDNF-AS* rs6265 association with BMD has not been explored in athletic cohorts so these findings could suggest that the GG genotype may be disadvantageous for BMD in this population and contribute towards the low LSBMD reported in some endurance runners (Pollock et al., 2010). It is difficult to suggest a



potential mechanism for this finding as osteoblastic cells transfected with the A allele have shown decreased BDNF protein phosphorylation, expression of osteoblastic genes and osteoblastic activity, whilst lower BMD has also been reported in those possessing the A allele in humans (Deng et al., 2013). It therefore could also be postulated that runners are able to combat a potential disadvantageous genetic predisposition via high volumes of mechanical loading and still reach high-level athlete status. This hypothesis, however, needs to be interpreted with caution as it would be expected to occur at the sites of greatest loading and no genotype-cohort interaction was observed for LBMD in this chapter. Moreover, limited study exists in regards to potential *BDNF-AS* associations with BMD and further investigation in both functional and association studies is required to provide greater evidence regarding mechanism and outcome for BMD.

No further genotype-cohort interactions for any bone phenotypes were observed in either the male or female analysis. The findings in regard to the *P2RX7* rs3751143 and *TNFRSF11A* rs3018362 SNPs are therefore, in agreement with previous study, which found no genotype by time interactions on bone phenotypes following completion of a 12-week training programme in academy football players (Varley et al., 2018). All the participants in the Varley et al. (2018) study were over the age of 16 years and it could be suggested that the majority of age-associated bone accrual may have been attained by the footballers by this age, or that the 12-week training period may not be long enough to observe changes in the bone phenotypes measured in some participants. Interestingly, the SNPs investigated in the Varley et al. (2018) study exhibited varying associations on the different bone phenotypes. For example, *P2RX7* rs1718119 was associated with cortical thickness, whereas *SOST* rs1877632 was associated with trabecular density. Cortical bone adapts to loading at a slower rate than trabecular bone, which may be due to genotype-dependent differences. Certain SNPs may be

sensitive to particular types of mechanotransduction, which could consequently effect the associated molecular pathway resulting in these differences in bone phenotypes as described in Chapter 4 in relation to *LRP5*. Potential genotype-dependent differences in responses to various types of mechanical loading may explain why inconsistency in the literature in regards to gene-mechanical loading and outcomes for bone phenotypes exists.

Of the other investigated variants in this chapter, *AXIN1* rs9921222, *LRP5* rs3736228 and *VDR* rs2228570 have been reported to have potential gene-physical activity/mechanical loading interactions with BMD previously (Mitchell et al., 2016; Nakamura et al., 2002b). Observing no gene-cohort (physical activity) interaction for *AXIN1* rs9921222, *LRP5* rs3736228 or *VDR* rs2228570 in this chapter may be due to differences in the investigated populations and/or measurement of physical activity. Participants in this investigation were high-level endurance runners who had experiences large amounts of mechanical loading during training to achieve the personal best criteria as highlighted in Chapter 2. Mitchell et al. (2016), however, utilised questionnaires to measure physical activity level in children, which can present issues with accuracy and reliability (Prince et al., 2008), particularly in non-adult populations, whilst Nakamura et al. (2002b) investigated track and field as well as handball and volleyball athletes. The track and field athletes comprised high and long jumpers, which alongside handball and volleyball constitute movements of high-impact loads and forces that are multi-directional in nature and thus are very different to the lower impact and cyclical movements completed in endurance running. These differences in movements may affect the signalling pathways differentially and the outcomes of potential gene-mechanical loading interactions for bone may be varied as a result as discussed above. Furthermore, most studies have been completed in adult populations and it is suggested some BMD-associated loci may exert age-specific

effects (Medina-Gomez et al., 2012), and thus, the findings from the Mitchell et al. (2016) study cannot be generalised across different population groups.

### *Genotype associations*

Genotype associations with BMD were present for the *COL1A1* rs1800012, *P2RX7* rs3751143 and *TNFRSF11A* rs3018362 variants in this chapter. *COL1A1* rs1800012 genotype explained >8.1% of the variance in LBMD and T-score in the total male cohort, with individuals who were AA genotype possessing higher LBMD and T-score than those who were AC genotype. Possessing the A allele of the *COL1A1* rs1800012 SNP is associated with reduced amount of transcript for  $\alpha 1$  chain, resulting in the formation of collagen homotrimers, which are associated with degenerative bone microarchitecture (Mann et al., 2001; Grant et al., 1996; Dytfield et al., 2016). This mechanism may explain why males with the AA genotype possessed higher BMD in the current chapter, however, it does not account for why this was not observed in females. Moreover, meta-analysis in untrained adult populations has reported that AA genotype is associated with higher BMD at a number of anatomical sites and reduced risk of fracture in females only (Jin et al., 2011; Ralston et al., 2006). No apparent *COL1A1* rs1800012 association with BMD/fracture in males via meta-analysis may be due to the research focus on osteoporosis, consequently resulting in fewer studies on males and therefore, further study is required to confirm whether the *COL1A1* rs1800012 influence is sex-dependent. Sex-variant interactions have been reported in some BMD-associated genetic loci in children, which highlights this could also be the case for *COL1A1* rs1800012 (Mitchell et al., 2015).

*TNFRSF11A* rs3018362 was associated with TBMD, but similar to *COL1A1* rs1800012, in males only. Those of GG genotype possessed ~4% higher TBMD than those of GA genotype in the total cohort. Similarly in the non-athletes, GG genotypes possessed higher TBMD than those of GA genotype. In the dominant analysis model,

GG genotypes possessed higher TBMD than A allele carriers in both the non-athletes and total cohort analysis. Binding of RANK (*TNFRSF11A*) to RANKL on the surface of osteoclast precursors initiates a large number of signalling pathways, such as Mitogen activated protein kinase (MAPK), resulting in activation of osteoclasts (Wada et al., 2006). The specific nature of how *TNFRSF11A* genotype differences may influence bone metabolism is not well understood due to the number of signalling cascades that induce the activation of bone metabolism transcription factors during RANK to RANKL ligation (Atkins et al., 2006). However, it could be proposed that RANK expression could be increased by possessing the GG genotype, which results in facilitation of RANK-RANKL binding and consequently, greater initiation of the signalling cascades as well as increased osteoclastogenesis.

The findings from this chapter suggest that possessing the *TNFRSF11A* rs3018362 GG genotype may be important for BMD in non-athlete populations as suggested by previous GWAS and candidate gene association study, where the A allele has been associated with lower BMD (Paternoster et al., 2010; Styrkarsdottir et al., 2009). No *TNFRSF11A* rs3018362 association with TBMD in the runners was exhibited, which could indicate that high-level runners may be able to compensate for a potential disadvantageous genetic predisposition for *TNFRSF11A* rs3018362. Large volumes of mechanical loading could result in increased BMD and provide greater security against stress fracture incidence despite a potential disadvantageous predisposition. This potential protective mechanism for BMD has been observed in children, where the benefit of weight-bearing physical activity on BMD was still observed in those with a disadvantageous genetic predisposition (Mitchell et al., 2016).

*P2RX7* rs3751143 was associated with differences in bone phenotypes in females but not males. Specifically in the total cohort, those of AA genotype possessed higher TBMD, LBMD, T-score and Z-score than those of AC genotype. AA genotypes also

possessed 4% higher TBMD and LBMD than C allele carriers in the dominant analysis model. These findings suggest that the AA genotype is associated with higher BMD as congruent with previous investigation (Wesselius et al., 2013). Wesselius et al. (2013) observed decreased hip BMD values in CC genotype women over the age of 50 years, whilst another investigation reported the CC genotype to be associated with increased fracture risk (Ohlendorff et al., 2007). Lower LSBMD in CC genotypes has been observed in osteoporotic females (Husted et al., 2013) although no association was observed in this chapter, which could again be due to the differences in the populations investigated, the influence of mechanical loading or the confounding effects of age. The *P2RX7* rs3751143 variant has been shown to have effects on purinergic receptor P2X 7 functioning and mice with a null mutation of *P2RX7* have >73% reduced sensitivity to mechanical loading (Li et al., 2005). Consequently, genetic variation in *P2RX7* SNPs such as rs3751143 could produce differing responses of BMD to mechanical loading.

Although no genotype-cohort interaction on any bone phenotypes was observed for the *P2RX7* rs3751143 to highlight this potential mechanism, genotype-dependent differences in bone phenotypes were apparent within the runners. Z-score was higher in the AA than AC genotypes, with a tendency for AA genotypes to possess higher TBMD and T-score than AC genotypes. Moreover, AA genotypes possessed higher LBMD than C allele carriers, albeit not statistically significant, in the dominant model analysis. These findings indicate that possessing the AA genotype might aid in increasing BMD (particularly at the running site-specific loading sites) and consequently protect from stress fracture. Although BMD was not measured, Varley et al. (2016) observed associations between the *P2RX7* rs3751143 C allele and stress fracture incidence in elite athletes. *P2RX7* rs3751143 has been proposed to work in a dose-response manner, with possession of the null CC genotype resulting in a loss of

receptor function, whereas heterozygotes are observed to have half the receptor functioning (Gu et al., 2001). The *P2RX7* rs3751143 C allele has been associated with osteoclast apoptosis (Ohlendorff et al., 2007) and reduced bone strength (Varley et al., 2018) which indicates that the loss-of-function C allele may reduce BMD and increase risk of stress fracture. It would therefore be expected that CC genotypes would possess the lowest BMD in the runners in the current study. However, a tendency for AC genotypes to possess lower TBMD and T-score than CC genotypes was observed, whilst CC genotypes possessed the highest TBMD in the runners. Despite this observation, only a small number of female runners possessed the CC genotype (n = 3) and runners with the AA genotype did possess the highest BMD at the site of higher stress fracture incidence in runners (LBMD). Moreover, AA genotypes possessed higher LBMD than C allele carriers in the dominant model analysis as mentioned above. Consequently, when taking all of the above findings into consideration, the results indicate a potential genotype-mediated protective mechanism against stress fracture incidence for runners via AA genotype and enhanced BMD.

The findings for *P2RX7* rs3751143 and the other significant variant associations in this chapter, however, have to be interpreted with caution. Bone phenotype data for each variant genotype, particularly rare homozygotes, is based on a small sample of high-level endurance runners. This population, however, represent one of the most appropriate to investigate BMD and potential gene-physical activity interactions and thus, further study to explore these associations is certainly warranted.

This study is the first to investigate BMD-associated variants in a homogenous group of high-level endurance runners in comparison with a non-athlete control group. Runners who possessed the *BDNF-AS* rs6265 GG genotype possessed a lower LSBMD than non-athletes who possessed GG genotype, which suggests the

advantageous (higher BMD-associated) G allele may in fact contribute to lower LSBMD in endurance runners. Runners with the *WNT16* rs3801387 (lower BMD-associated) AA genotype possessed lower LSBMD and Z-score, and a tendency to possess lower LBMD, than non-athletes who were AA genotype. Additionally, a tendency for runners with AG genotype to possess higher LBMD than non-athletes with AG genotype was also observed, highlighting an interaction between genotype and the two cohorts for the two anatomical sites. These findings could suggest that the G allele is important for BMD in male endurance runners via a mechanical loading interaction. *TNFRSF11A* rs3018362 GG and *COL1A1* rs1800012 GG genotypes are associated with higher TBMD and LBMD in males, respectively, whilst *P2RX7* rs3751143 AA genotype may be important for BMD in female endurance runners, regardless of mechanical loading. Further study in large sample sizes of homogenous athletic populations such as endurance runners is required to investigate the genetic association with BMD and provide greater evidence for these findings. Limited study into the genetics of BMD in athletes exists and utilising homogenous athletic populations will aid in exploring potential gene-mechanical loading interactions, which could have substantial implications for skeletal health and injury.

## **Chapter 6:**

# **Bone mineral density and stress fracture occurrence in high-level endurance runners**



## 6.1 Introduction

Stress fractures are defined as a partial or complete fracture of bone from repeated application of force lower than that required to fracture a bone in a single loading (Iwamoto and Takeda, 2003). Stress fracture injury occurs due to the repetitive mechanical loading that stimulates an incomplete or dysfunctional remodelling response and exceeds intrinsic repair mechanisms (Bennell et al., 1999). Specifically, under continued extreme volumes of mechanical loading without an ample rest period, a micro-crack may be induced. Subsequently, increased intra-cortical remodelling via enhanced osteoblastic activity resulting in bone expansion and reduced volume may occur (O'Brien et al., 2003; Burr et al., 1997; Doblaré et al., 2004). Several factors are known to influence an individual's susceptibility to experiencing a stress fracture and include biomechanical gait (Milner et al., 2006), bone size and mechanical properties (Tommasini et al., 2005), nutritional factors (Nieves et al., 2010), genetics (Korvala et al., 2010), training volume and rapid increments in volume (Snyder et al., 2006), small musculature and low bone mineral density (BMD) (Beck et al., 2000).

A substantial proportion of the research investigating stress fracture determinants, such as BMD, has been conducted in military recruits. It is difficult to extrapolate the findings of these military studies to other populations, such as athletes, due to differences in the level of physical fitness, footwear and the loads carried whilst running between these groups (Wright et al., 2015). Many of these studies have also used inappropriately matched control groups that may differ in other risk factors, such as body mass, which means it is difficult to analyse the extent of the association of the investigated risk factor. For example, although lower BMD was present in male recruits who developed a stress fracture, these recruits were also 11% lighter than those who had not suffered a stress fracture (Beck et al., 1996; Bennell et al., 1999).

Stress fracture prevalence can be significant in athletic populations due to the repetitive mechanical loading undertaken and this can have negative consequences for health and performance. If athletes are unable to complete their desired or required training volume due to injury, this could have substantial negative effects on their performance and subsequent success. In endurance runners, higher incidence of lower limb stress fractures is observed in comparison with non-athletic controls, reportedly accounting for 15-20% of all musculoskeletal injuries sustained by runners, with higher incidence observed in females (Milner et al., 2006; Wright et al., 2015). Significant amounts of site-specific loading combined with other factors typical of this group, such as low energy availability and low body mass, can result in lower BMD and a higher risk of fracture occurrence (Loucks, 2007). Despite this, meta-analysis demonstrated there is only data to suggest previous history of stress fracture and female sex as the two key risk factors for stress fractures in endurance populations (Wright et al., 2015). There is, however, a lack of robust research into the other, aforementioned, potential risk factors for stress fractures. In particular, the possible influence of BMD on stress fracture risk in endurance runners requires further investigation. Both male and female endurance runners have been shown to possess low BMD or lower BMD than that of non-athletes, particularly at non-loading sites (Hind et al., 2006; Pollock et al., 2010). Similarly, research so far has suggested this low or lower BMD in endurance runners is associated with stress fracture but this conclusion is based on a limited number of conflicting studies.

Low total bone mass has been associated with stress fracture risk in collegiate/postcollegiate female cross-country runners aged 18–26 years old (Kelsey et al., 2007) and female track and field athletes, in addition to lower lean mass, leg-length discrepancy and fewer menstrual cycles per year over a 12-month period, but not males (Bennell et al., 1996). Crossley et al. (1999) also reported no association

with tibial BMD and stress fracture in male endurance runners, whereas Nattiv et al. (2000) observed lower total-body, spine and hip BMD in both male and female collegiate runners who developed stress fractures in comparison to their uninjured counterparts. Low BMD, therefore, is suggested to be a risk factor for stress fracture in runners, at least in females, although contrasting findings have been reported. Korpelainen et al. (2001) reported no difference in BMD between six female long-distance runners who had suffered at least one stress fracture and a control group, whilst Duckham et al. (2012) and Duckham et al. (2015) also observed no association between BMD and stress fracture injury in UK high-level female endurance runners and triathletes.

Alongside the lack of research investigating BMD and stress fracture incidence in endurance runners, the few studies on this topic have not always measured the specific site of the stress fracture, thus, the BMD data reported may not be reflective of the fracture site BMD (Bennell et al., 1999). Additionally, studies in the field investigating BMD and stress fracture association have used small sample sizes and comprise athletes of differing training volumes and ability. Duckham et al. (2012) and (2015) utilised triathletes and runners across a range of abilities from county to international level and observed only two runners who suffered a stress fracture across the 12-month prospective study. This lack of homogeneity and dissimilarity in both the participants used and the methodological design are likely to increase the inter-individual variability within the phenotype through differences in training characteristics and mechanical loading as outlined in Chapter 3 of this thesis. Overall, little research exists to determine the association of BMD on stress fracture, particularly in homogenous cohorts of high-level endurance runners.

Further research is needed to explore stress fracture injury in running populations. Stress fracture injury can have a substantial negative impact on training, performance

and health, ultimately influencing the likelihood of achieving elite status. Utilising large sample sizes of high competitive level (i.e. national and international) endurance runners is fundamental to extending our understanding of the importance of BMD on stress fracture incidence in this population. Effective prevention strategies can then be developed and implemented to reduce risk and ultimately enhance performance. The aim of this study, therefore, was to assess total BMD (TBMD), leg BMD (LBMD), lumbar spine BMD (LSBMD), total-body T-score and Z-score of high-level endurance runners who had suffered a stress fracture in comparison to those who had not.

## **6.2 Method**

The investigated participants and protocols used in Chapter 6 have already been described in detail in Chapter 2, thus, only a brief description of these methods is detailed below.

### *Participant characteristics*

Participants consisted of 103 high-level endurance runners (45 males, 58 females) as described in Table 2.1 of Chapter 2. To ensure subsequent analyses occurred between clearly defined participant groups and explored BMD in runners with other potential bone-overloading conditions: Participants who had a stress fracture diagnosed by a scan were placed in the stress fracture (SF) group and those who reported no history of stress fracture were placed in the no stress fracture (NSF) group. Those participants who reported a stress response/stress reaction via scan or reported having a potential stress fracture diagnosed by a medical professional were classified in the symptoms of stress fracture (SSF) group. The SSF group contained runners who had scan diagnoses confirming a stress response/reaction and not a fracture as well as those who were hypothesised to have a potential fracture based on symptoms by a medical professional and therefore, fracture occurrence had no scan confirmation.

Dividing participants in this manner removed runners that lacked stress fracture clarity, thus, allowing for analysis between clearly defined groups. Of the 103 runners, 7 males and 15 females had suffered at least one stress fracture injury (SF), 36 males and 32 females had no history of stress fracture injury (NSF) and 2 males and 11 females reported a stress response/stress reaction via scan or stress fracture diagnosis by a medical professional (SSF).

### *Protocol*

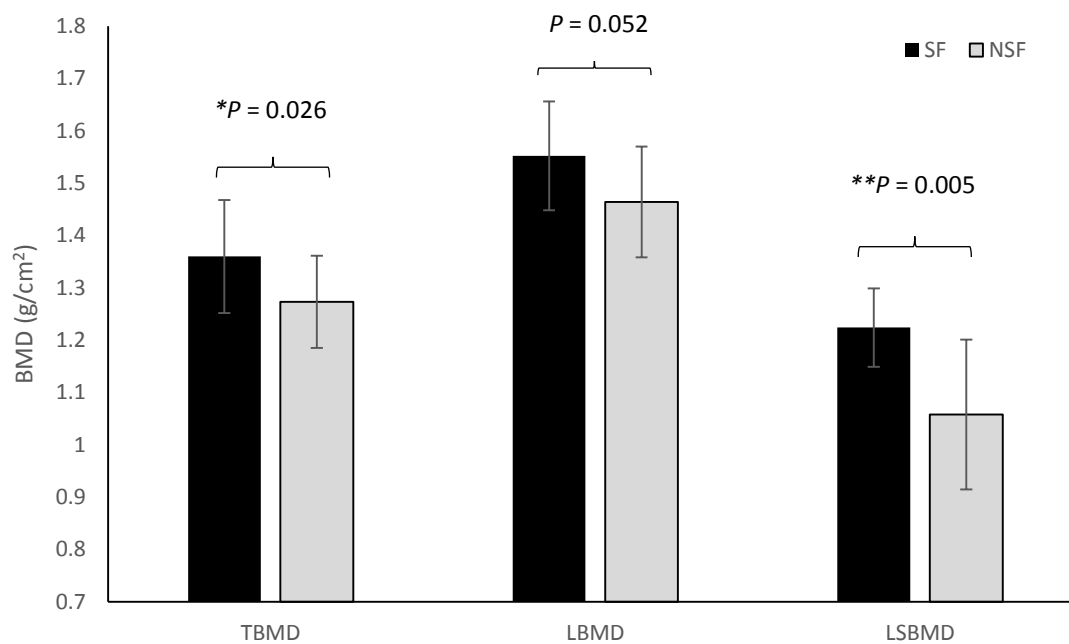
All runners completed a questionnaire detailing ethnic ancestry, as well as performance, injury and sporting history (Appendix 1). Stress fracture injury was self-reported and runners were asked to confirm whether their stress fracture had been diagnosed by computed tomography (CT) or magnetic resonance imaging (MRI) scan on the questionnaire, and/or if the stress fracture had been diagnosed by a medical professional. All participants completed one whole-body DXA scan to obtain BMD data with whole-body and segmental analysis utilised to gather total-body BMD (TBMD), leg BMD (LBMD), lumbar spine BMD (LSBMD), total-body T-score and Z-score.

### *Statistical analysis*

Multiple analysis of variance (MANOVA) was used to compare TBMD, LBMD, LSBMD, T-score and Z-score between the SF and the NSF groups of the female runners and male runners independently. MANOVA on the same phenotypes was then completed on the SF group, SSF group and NSF group in the female runners only before Bonferroni correction were implemented to account for multiple-testing. In the male analysis, only two runners were placed into the SSF group so no further sub-group analyses were completed. Alpha was set at 0.05 and data were reported as mean (SD) unless otherwise stated.

### 6.3 Results

TBMD ( $P = 0.026$ ), and LSBMD ( $P = 0.005$ ) were ~7%, and ~16% higher, respectively, in SF than NSF male runners but no difference in LBMD ( $P = 0.052$ ) between the two groups was observed (Figure 6.1; Table 6.1). Total-body T-score ( $P = 0.028$ ) and Z-score ( $P = 0.034$ ) were also higher in the SF than NSF group (Table 6.1).



**Figure 6.1:** Differences in TBMD (\* $P = 0.026$ ) and LSBMD (\*\* $P = 0.005$ ) but not LBMD ( $P = 0.052$ ) between SF and NSF male runners. Error bars denote standard deviation.

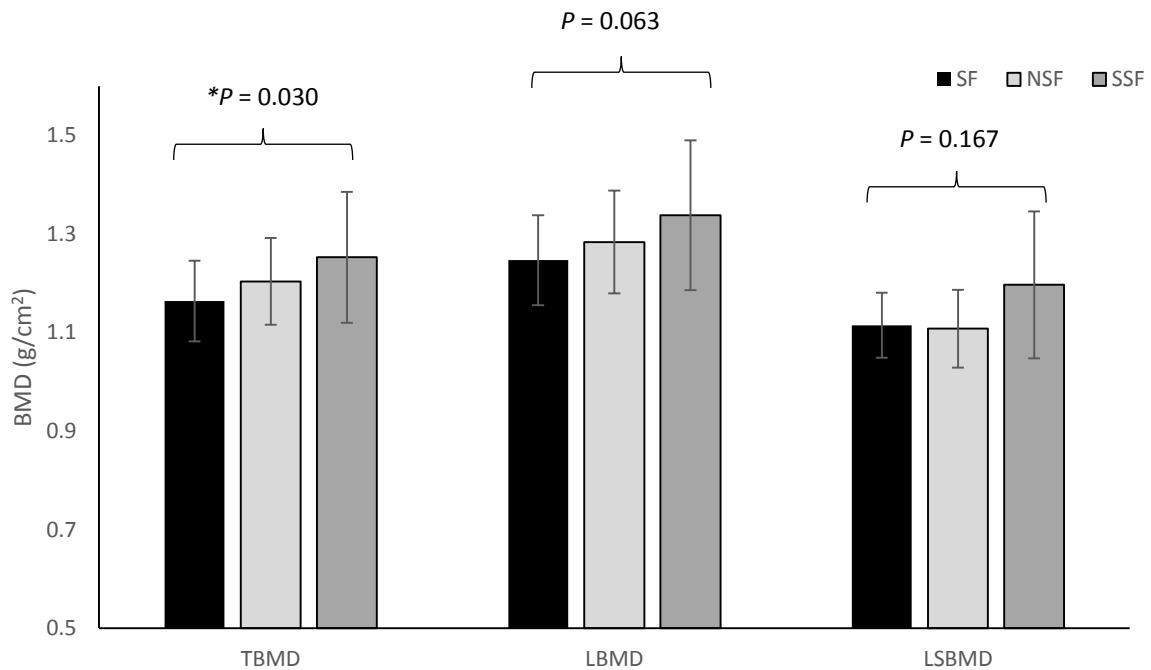
**Table 6.1:** Anthropometric and BMD phenotype data in SF (n = 7) and NSF male runners (n = 36). Data are presented as mean (SD) except for TBMD, LBMD, LSBMD, T-score and Z-score which are presented as mean (minimum-maximum).

	<b>SF</b>	<b>NSF</b>
Age (years)	38 (11)	36 (9)
Height (m)	1.80 (0.06)	1.78 (0.06)
Mass (kg)	70.609 (5.041)	66.401 (6.835)
TBMD (g/cm <sup>2</sup> )	<b>1.360 (0.108)*</b>	1.273 (0.088)
LBMD (g/cm <sup>2</sup> )	1.552 (0.104)	1.464 (0.106)
LSBMD (g/cm <sup>2</sup> )	<b>1.224 (0.075)*</b>	1.058 (0.143)
T-score	<b>1.53 (0.93)*</b>	0.73 (0.84)
Z-score	<b>1.46 (0.88)*</b>	0.71 (0.82)
TBMD Range	0.296 (1.226–1.522)	0.434 (1.034–1.468)
LBMD Range	0.257 (1.438–1.695)	0.526 (1.193–1.719)
LSBMD Range	0.230 (1.150–1.380)	0.810 (0.750–1.560)
T-score range	2.60 (0.30–2.90)	4.20 (-1.70–2.50)
Z-score range	2.50 (0.20–2.70)	4.00 (-1.60–2.40)

\*Indicates differences from NSF group

No differences in TBMD ( $P = 0.154$ ), LBMD ( $P = 0.236$ ), LSBMD ( $P = 0.870$ ), T-score ( $P = 0.152$ ) or Z-score ( $P = 0.143$ ) were observed between the female SF and NSF groups in the initial comparison (Figure 6.2; Table 6.2).

When analysed as three groups (SF, SSF and NSF), differences were only observed for TBMD, T-score and Z-score between the SF and SSF group. Higher TBMD ( $P = 0.030$ ), T-score ( $P = 0.033$ ) and Z-score ( $P = 0.015$ ) were possessed by the SSF female runners in comparison to the SF group (Figure 6.2; Table 6.2). No further differences were found for any BMD parameter between any group comparison ( $P \geq 0.057$ ; Figure 6.2; Table 6.2).



**Figure 6.2:** Differences in TBMD ( $*P = 0.030$ ) but not LBMD ( $P = 0.063$ ) or LSBMD ( $P = 0.167$ ) between the female SF and SSF runners. Error bars denote standard deviation.

**Table 6.2:** Participant anthropometric, BMD phenotype data, T-score and Z-score in SF ( $n = 15$ ), SSF ( $n = 11$ ) and NSF ( $n = 32$ ) female runners. Data are presented as mean (SD) except for TBMD, LBMD, T-score and Z-score which are presented as mean (minimum-maximum).

	SF	NSF	SSF
Age (years)	32 (11)	36 (13)	34 (12)
Height (m)	1.64 (0.05)	1.65 (0.06)	1.67 (0.06)
Mass (kg)	51.764 (4.478)	52.766 (5.642)	55.017 (4.389)
TBMD (g/cm <sup>2</sup> )	1.164 (0.082)	1.204 (0.091)	<b>1.253 (0.066)*</b>
LBMD (g/cm <sup>2</sup> )	1.247 (0.088)	1.284 (0.104)	1.338 (0.079)
LSBMD (g/cm <sup>2</sup> )	1.115 (0.133)	1.108 (0.152)	1.197 (0.149)
T-score	0.69 (0.99)	1.17 (1.07)	<b>1.74 (0.74)*</b>
Z-score	0.64 (0.85)	1.05 (0.89)	<b>1.63 (0.69)*</b>
TBMD Range	0.282 (1.036–1.318)	0.338 (1.010–1.348)	0.189 (1.171–1.360)
LBMD Range	0.354 (1.046–1.400)	0.415 (1.029–1.444)	0.282 (1.215–1.497)
LSBMD Range	0.420 (0.880–1.290)	0.760 (0.710–1.460)	0.370 (1.020–1.390)
T-score range	3.40 (-0.90–2.50)	4.00 (-1.20–2.80)	2.10 (0.80–2.90)
Z-score range	3.00 (-0.90–2.10)	3.10 (-0.80–2.30)	2.20 (0.60–2.80)

\*indicates differences from SF group



## 6.4 Discussion

The aim of this chapter was to investigate TBMD, LBMD, LSBMD, total-body T-score and Z-score in high-level endurance runners who had suffered at least one stress fracture in comparison to runners who had never suffered a stress fracture. No difference in BMD was observed in female runners who had suffered a stress fracture (SF) in comparison to those who had no stress fracture injury history (NSF). Lower BMD, however, was observed in SF female runners in comparison to those who had reported stress fracture symptoms but had no scan diagnosis (SSF). In male runners, the SF group possessed higher BMD than the NSF group. Consequently, this chapter is consistent with previous research suggesting that female endurance runners who have suffered at least one stress fracture may possess lower BMD than those who have no stress fracture injury history confirmed via scan but this is only apparent in those who have suffered stress fracture symptoms (SSF).

Low BMD can contribute to stress fracture incidence through reducing bone strength, resulting in increased accumulation of microdamage via repetitive loading over a prolonged time period (Bennell et al., 1999). It is interesting to note that when the runners were split into the three subgroups (SF, SSF and NSF), the SSF group possessed higher TBMD, LBMD, LSBMD, T-score and Z-score than the other two cohorts (albeit only significant for TBMD, T-score and Z-score between SF and SSF groups). The SSF group comprised runners who self-reported a medical professional diagnosed stress fracture/stress response/stress reaction or stress response/reaction via scan. It could be hypothesised that some of these SSF runners may not have suffered a true stress fracture (visible cortical fracture) due to possessing higher BMD. Possessing higher BMD may have provided greater protection to extensive loading and thus, explain why some only experienced stress responses/reactions and not a true stress fracture diagnosed via scan. Determining accurate prevalence of stress

fractures, however, is difficult due to the problematic nature of defining stress fractures and, therefore, some of the SSF may have suffered a stress fracture occurrence. Significant misdiagnosis of stress fractures can occur through medical professional assessment unless supported by radiography, although radiography can still lack sensitivity and specificity (Wright et al., 2016).

The notion that BMD was not higher in the NSF than SF group suggests that other factors, such as training characteristics, energy availability and/or menstrual dysfunction may also be influencing stress fracture susceptibility in female runners. No difference in BMD was observed between potential amenorrheic and eumenorrheic runners in Chapter 3 of this thesis, with previous studies having reported an association between menstrual irregularity and low BMD (Tenforde et al., 2015). The influence of energy availability and/or menstrual dysfunction, therefore may explain why some previous studies have reported BMD associations with stress fracture (Kelsey et al., 2007; Bennell et al., 1996) but this chapter did in the SF vs SSF but not SF vs NSF comparisons. It is interesting to note that the difference in BMD found between the female SF and SSF groups was total-body and not site-specific (i.e. leg). Although LBMD provides better analysis than TBMD to assess the influence of BMD when the fracture sites are present in the lower extremity (Bennell et al., 1999), each standard deviation decrease in TBMD (293.2 g) in female endurance runners, has been reported to increase stress fracture rate two-fold (Kelsey et al., 2007). It is also important to emphasise that LBMD was still lower in the SF group than the NSF group, albeit non-significant. Possessing low BMD increases the potential risk of stress fracture but leg geometry, hormonal and nutritional factors are also contributory risk factors (Moreira and Bilezikian, 2016) and thus, possessing low BMD does not confirm a runner will suffer a stress fracture. The same principle applies with osteoporosis, where individuals with low BMD and consequently, diagnosed with the condition, may

never suffer an osteoporotic fracture, as other factors such as bone geometry, collagen properties and microarchitecture also influence clinical risk (Schoenau et al., 2002; Fonseca et al., 2014; Cheung et al., 2016).

In the male analysis, higher TBMD, LSBMD, T-score and Z-score were possessed by the SF group in comparison to the NSF group, whilst no differences in LBMD were observed between the two groups. Bennell et al. (1996) and Crossley et al. (1999) both reported no association with BMD and stress fracture risk in male runners, although BMD was not higher in their respective stress fracture groups. In male runners, it would therefore appear that other risk factors, such as anthropometrics, gait, genetics and rapid changes in training are potentially more important than BMD for stress fracture susceptibility. Body mass has been proposed as a significant determinant for stress fracture risk in military recruits (Knapik et al., 2012) but no differences in body mass or height were present between the SF and NSF group in this chapter. Kinematic and kinetic variables such as peak hip adduction, peak rear foot eversion and absolute free movement have also been reported to predict tibial stress fracture history (Pohl et al., 2008). More recently, genetic associations with stress fracture have also been reported in athletes (Varley et al., 2015; Varley et al., 2016; Varley et al., 2017). Genetics and running gait are likely to have substantial variance in endurance running populations, and although not investigated in this chapter, may have provided greater aetiology, rather than BMD, to stress fracture occurrence in the male runners in this chapter. The potential finding that BMD is not influencing stress fracture incidence in males, however, must be taken with caution. Only seven male runners had suffered a stress fracture in this investigation and a very small number of studies investigating stress fractures and BMD in male endurance runners exist with some reporting conflicting findings to this chapter, suggesting a potential association with low BMD and stress fracture occurrence (Bennell et al., 1996; Crossley et al., 1999; Nattiv et al., 2000).

Further investigation of BMD in male runners with stress fractures is required before confident interpretation regarding the influence of BMD on stress fractures can be concluded. In Chapter 3 of this thesis, lower LSBMD was observed in the male endurance runners in comparison to controls, as also reported in previous studies (Hind et al., 2006). Lower BMD has been reported in female runners with stress fractures alongside reduced energy availability, which can also occur in males (Mountjoy et al., 2018). Stress fracture occurrence is multi-factorial and it is likely that a combination of potential factors discussed throughout this chapter will contribute to stress fracture risk.

Runners were included as part of the SF group as long as they had suffered at least one stress fracture incidence and thus, some may have had stress fractures that occurred a substantial amount of time ago. Variation between participants in the time between undergoing the DXA scan, when the stress fracture occurred and at which anatomical site, as well as in the severity of the stress fracture may influence the findings due to the likely disparity in the treatment programmes. It is possible some may have undergone extensive immobilisation or experienced a large reduction in mechanical loading volume, which may have had a modest effect on BMD (Bennell et al., 1999). Although this can be considered a limitation of the study, the vast majority of stress fractures in runners heal within 8 weeks through conservative treatment (Kahanov et al., 2015) and the mean age of the runners was 34 in the females and 36 in the males. BMD remains relatively stable (unless severe and/or prolonged unloading occurs) after peak bone mass is reached up until aged 50 approximately (Bonjour et al., 1994).

This chapter suggests that BMD does not appear to influence stress fracture incidence in male endurance runners and thus, other determinants such as gait, training characteristics or genetics may contribute to stress fracture occurrence. Female

runners, however, who had symptoms but did not suffer a stress fracture confirmed via scan appear to possess higher TBMD, T-score and Z-score than those who have suffered a stress fracture diagnosed via scan. Such higher BMD might protect against development of a confirmed stress fracture despite continued extensive loading during training.

## **Chapter 7:**

# **Genetic associations with stress fracture occurrence in high-level endurance runners**

## 7.1 Introduction

Stress fractures are defined as a partial or complete fracture of bone from repeated application of force lower than that required to fracture a bone in a single loading (Iwamoto and Takeda, 2003). Stress fracture injury occurs due to the repetitive mechanical loading that stimulates an incomplete remodelling response, exceeding intrinsic repair mechanisms (Bennell et al., 1999). Several factors are known to influence an individual's susceptibility to experiencing a stress fracture and include biomechanical gait (Milner et al., 2006), bone size and mechanical properties (Tommasini et al., 2005), nutritional factors (Nieves et al., 2010), training volume and rapid increments in training volume (Snyder et al., 2006), small musculature and low bone mineral density (BMD) (Beck et al., 2000).

The notion of a potential genetic influence on stress fracture susceptibility has also been proposed, with the majority of research having been completed in military recruits (Lappe et al., 2008). Indeed, the calcitonin receptor (*CTR*) rs1801197 and *LRP5* rs2277268 polymorphisms have been associated with femoral neck stress fractures in 72 Finnish military recruits and those recruits who possessed the *CTR* C allele together with a *VDR* rs10735810 C-A haplotype were more protected from stress fractures (Korvala et al., 2010). Furthermore, larger sized CAG androgen receptor gene repeats (>16) were more common in Israeli military personnel who had suffered stress fractures (23%) than those who had not suffered this injury (13%) (Yanovich et al., 2011). These findings, therefore, demonstrate there appears to be a genetic influence on stress fracture incidence in populations of increased training/loading.

Athletes and in particular, endurance runners, are another cohort where high incidence of stress fractures are reported, accounting for 15-50% of all injuries (Milner et al., 2006). Stress fractures can have substantial negative implications for health and

performance in endurance runners, potentially affecting training load/volume and consequently providing a barrier to success or reaching high performance levels. Although meta-analysis has suggested that only previous history of stress fracture and female sex are the two key risk factors for stress fracture incidence in endurance runners (Wright et al., 2015), low BMD still remains a logical and supported risk factor for stress fracture susceptibility in this population (Kelsey et al., 2007; Nattiv et al., 2000; Bennell et al., 1996).

It could also be speculated that a lack of research on genetics associations with BMD is why only previous stress fracture history and female sex were the key two risk factors associated with stress fracture incidence. Heritability of BMD is suggested to be 50-85% (Ralston and Uitterlinden, 2010), depending upon anatomical location, whilst potential gene-mechanical loading interactions influencing BMD in athletes have also been reported (Nakamura et al., 2002a; Nakamura et al., 2002b). Studying potential genetic associations with stress fracture in athletes due to the substantial heritable nature of BMD, therefore, provides an interesting and noteworthy route to further explore the aetiology of stress fracture susceptibility.

A limited number of studies have investigated a potential genetic association with stress fracture injury so far, all having been conducted in the Stress Fracture Elite Athlete (SFEA) cohort (Varley et al., 2015; Varley et al., 2016; Varley et al., 2017). *VDR* FokI rs2228570, *TNFSF11* (RANKL) rs1021188, *P2RX7* rs3751143 and *TNFRSF11A* (RANK) rs30218362 were some of the SNPs associated with stress fracture in the 125 athletes that had suffered a radiographically confirmed stress fracture in comparison to 376 athletes who had no stress fracture history. Investigation of the genetic association on stress fracture using the SFEA cohort, represents the largest study in athletes to date, consisting of 501 athletes in total. These athletes, however, were of mixed abilities from a range of sports, which varied in loading/training



patterns/physical characteristics, which have been shown to influence stress fracture susceptibility (Bennell et al., 1996; Cosman et al., 2013; Bennell et al., 1999). Utilising a homogenous group of athletes from one sport would therefore, somewhat alleviate differences in these aforementioned factors that influence stress fracture susceptibility and allow better assessment of genetic association of stress fracture injury. Greater understanding of the aetiology of stress fracture injury will ultimately allow better development and implementation of training, prevention and management strategies to aid in performance and recovery.

The aim of this study, therefore, was to compare the genotype and allele frequencies of the 10 genetic variants associated with BMD described in Chapter 1, in high-level runners who had suffered a stress fracture in comparison with those who had never suffered a stress fracture. It was hypothesised that the runners who had suffered a stress fracture would possess more “risk” (lower BMD associated) genotypes in comparison to the runners who had never suffered a stress fracture suggesting a potential genetic contribution to stress fracture injury in endurance runners.

## **7.2 Method**

The participants and protocols used in Chapter 7 have already been described in detail in Chapter 2, thus, only a brief description of these methods is detailed below.

### *Participants*

Participants consisted of 195 high-level Caucasian runners (102 males, 93 females) from the cohort as described in Table 2.1 of Chapter 2. To ensure subsequent analyses occurred between clearly defined participant groups, participants who had a stress fracture diagnosed by a scan were placed in the stress fracture (SF) group and those who reported no history of stress fracture were placed in the no stress fracture (NSF) group. Those participants who reported a stress response/stress reaction via scan or

reported having a stress fracture diagnosed by a medical professional but not confirmed via scan were classified into the symptoms of stress fracture group stress fracture (SSF) group. Dividing participants in this manner removed potential misdiagnoses of stress fractures and thus allowed for analysis between clearly defined groups. Consequently, a total no stress fracture group consisting of SSF + NSF (TNSF) were compared to the SF group before the SSF runners were removed and a comparison between the clearly defined groups was completed (SF vs NSF). Of the 195 runners, 12 males and 21 females had suffered at least one stress fracture injury (SF), 86 males and 56 females had no history of stress fracture injury (NSF) and 4 males and 16 females reported a stress response/stress reaction via scan or having a stress fracture diagnosed by a medical professional (SSF).

### *Protocol*

All runners completed a questionnaire detailing ethnic ancestry, as well as performance, injury and sporting history (Appendix 1). Stress fracture injury was self-reported and runners were asked to confirm whether their stress fracture had been diagnosed by computed tomography (CT) or magnetic resonance imaging (MRI) scan on the questionnaire, and/or if the stress fracture had been diagnosed by a medical professional. All participants provided a whole-blood, saliva or buccal swab sample, from which DNA was subsequently extracted and analysed to obtain genotype data for the 10 investigated SNPs (*AXIN1* 9921222, *BDNF-AS* rs6265, *COL1A1* rs1800012, *COMT* rs4680, *LRP5* rs3736228, *P2X7R* rs3751143, *TNFRSF11A* rs3018362, *TNFRSF11B* rs4355801, *VDR* rs2228570, *WNT16* rs3801387) as described in Chapter 2.

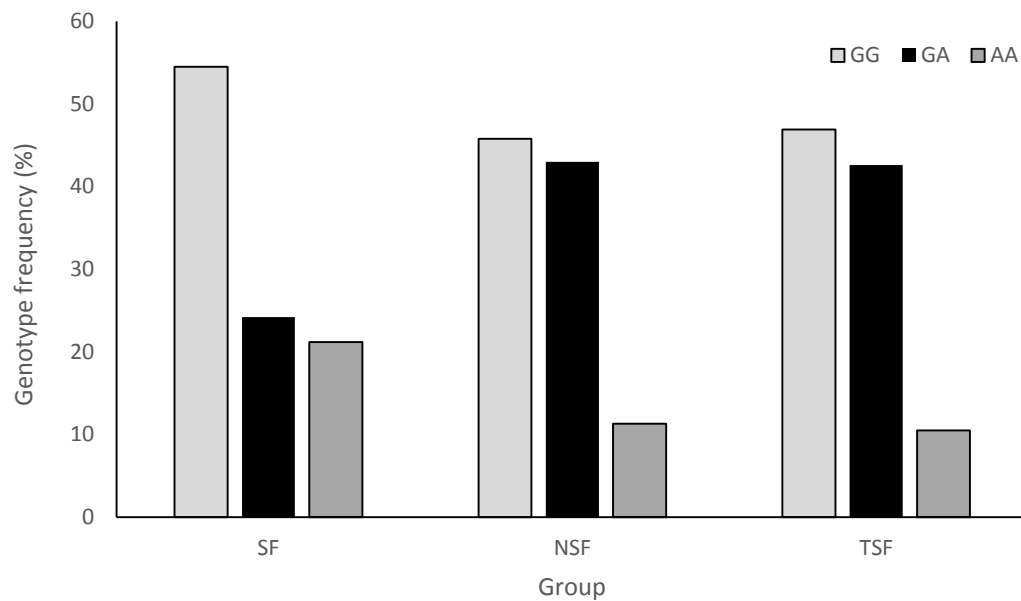
### *Statistical analysis*

Pearson's Chi-square ( $\chi^2$ ) tests were utilised to compare genotype (using three analysis models; additive, recessive and dominant) and allele frequencies between the SF and the combined NSF and SSF groups (TNSF) before being implemented between the SF and NSF groups. Fisher's exact test was used for genotype analysis when a genotype group had a sample size  $<5$  and  $\chi^2$  could not be completed, which occurred on 18 occasions. Odds ratios were also calculated to estimate effect size. Independent t-tests were completed to compare total genotype score (TGS) between SF and NSF as well as SF and TNSF groups. TGS was calculated and implemented in the same manner as outlined in chapter 4, where each SNP homozygote associated with higher BMD given a score of 2, the heterozygotes scoring 1 and the other homozygote given 0 ("risk" genotype). The total score was then calculated to lie within 0-100 ((e.g.  $TGS = 100/20 \times (2+1+0+1+1+1+0+2+1+2) = 55$ )). Receiver operator curve (ROC) area under the curve (AUC) analyses was conducted to determine if TGS was able to classify SF from NSF or TNSF runners. Benjamini-Hochberg corrections were implemented for genotype and allele frequency comparisons when required (each SNP submitted to 8 tests) to account for false discovery rate. Corrected probability values are reported and alpha was set at 0.05.

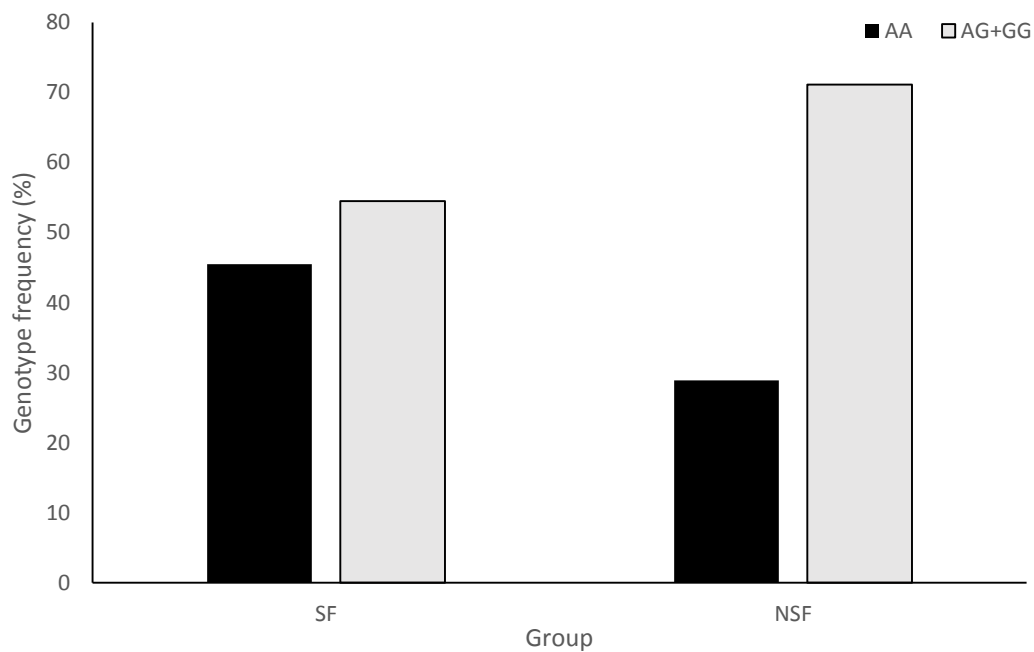
### **7.3 Results**

No differences in genotype or allele frequency were observed for any SNP between SF vs NSF or SF vs TNSF comparisons after multiple testing correction ( $P \geq 0.120$ ; Table 7.1). Before multiple testing correction, the *TNFRSF11A* rs3018362 "risk" (lower BMD-associated) AA genotype was more frequent in the SF than NSF group and in the SF vs TNSF group comparison (Figure 7.1), whilst in the recessive

analysis model, the *TNFRSF11B* rs4355801 “risk” (lower BMD-associated) AA genotype was more frequent in the SF than NSF group (Figure 7.2).



**Figure 7.1:** *TNFRSF11A* rs3018362 AA genotype in SF, NSF and TNSF runners.



**Figure 7.2:** *TNFRSF11B* rs4355801 AA and AG + GG genotypes in SF and NSF runners

**Table 7.1:** Genotype and allele frequencies of all investigated SNPs in SF, NSF, SSF and TNSF groups with their respective *P*- and  $\chi^2$  values from the additive analysis model. N/A indicates that no individual possessed the specific genotype for that SNP.

SNP	Genotype Allele	SF	NSF	SSF	TNSF	SF vs NSF	SF vs TNSF
		Genotype frequency (%)	Genotype frequency (%)	Genotype frequency (%)	Genotype frequency (%)	P-value	P-value
		Allele frequency (%)	Allele frequency (%)	Allele frequency (%)	Allele frequency (%)	Chi-square ( $\chi^2$ )	Chi-square ( $\chi^2$ )
<i>AXIN1</i> rs9921222	CC	30.3%	23.2%	50.0%	26.5%	0.390	0.540
	CT	42.4%	54.2%	35.0%	51.9%	1.885	1.232
	TT	27.3%	22.5%	15.0%	21.6%		
	C	51.5%	50.4%	67.5%	52.5%	0.850	0.977
	T	48.5%	49.6%	32.5%	47.5%	0.036	0.024
<i>BDNF-AS</i> rs6265	GG	63.6%	66.9%	65.0%	66.7%	0.546	0.565
	GA	36.4%	29.6%	30.0%	29.6%	N/A	N/A
	AA	0%	3.5%	5.0%	3.7%		
	G	81.8%	81.6%	80.0%	81.5%	0.979	0.944
	A	18.2%	18.3%	20.0%	18.5%	0.001	0.005
<i>COL1A1</i> rs1800012	AA	78.8%	62.7%	70.0%	63.6%	0.250	0.250
	AC	18.2%	33.1%	30.0%	32.7%	3.697	3.372
	CC	3.0%	4.2%	0.0%	3.7%		
	A	87.9%	79.2%	85.0%	80.0%	0.214	0.214
	C	12.1%	20.8%	15.0%	20.0%	3.003	2.595
<i>COMT</i> rs4680	GG	18.2%	22.5%	15.0%	21.6%	0.761	0.719
	GA	54.5%	48.6%	40.0%	47.5%	0.548	0.659
	AA	27.3%	28.9%	45.0%	30.9%		
	G	45.5%	46.8%	35.0%	45.4%	0.663	0.989
	A	54.5%	53.2%	65.0%	54.6%	0.190	<0.001
<i>LRP5</i> rs3736228	GG	69.7%	69.7%	85.0%	71.6%	0.987	0.967
	GA	27.3%	26.8%	15.0%	25.3%	0.026	0.067
	AA	3.0%	3.5%	0.0%	3.1%		
	G	83.3%	83.1%	92.5%	84.3%	0.959	0.836
	A	16.7%	16.9%	7.5%	15.7%	0.003	0.043
<i>P2X7R</i> rs3751143	AA	63.6%	62.7%	80.0%	64.8%	0.841	0.772
	AC	30.3%	33.1%	20.0%	31.5%	0.346	0.517
	CC	6.1%	4.2%	0.0%	3.7%		
	A	78.8%	79.2%	90.0%	80.6%	0.930	0.717
	C	21.2%	20.8%	10.0%	19.4%	0.008	0.132
<i>TNFRSF11A</i> rs3018362	GG	54.5%	45.8%	55.0%	46.9%	0.123	0.123
	GA	24.2%	43.0%	40.0%	42.6%	6.142	6.631
	AA	21.2%	11.3%	5.0%	10.5%		
	G	66.7%	67.3%	75.0%	68.2%	0.919	0.901
	A	33.3%	32.7%	25.0%	31.8%	0.010	0.072
<i>TNFRSF11B</i> rs4355801	AA	45.5%	28.9%	40.0%	30.2%	0.154	0.154
	GA	33.3%	52.8%	50.0%	52.5%	5.666	5.121
	GG	21.2%	18.3%	10.0%	17.3%		
	A	62.1%	55.3%	65.0%	56.5%	0.422	0.473
	G	37.9%	44.7%	35.0%	43.5%	1.249	0.854
VDR rs2228570	CC	30.3%	36.6%	55.0%	38.9%	0.229	0.190
	CT	42.4%	47.9%	40.0%	46.9%	3.521	4.741
	TT	27.3%	15.5%	5.0%	14.2%		
	C	51.5%	59.6%	75%	62.3%	0.213	0.190
	T	48.5%	39.4%	25%	37.7%	2.262	3.298
<i>WNT16</i> rs3801387	AA	45.5%	52.2%	65.0%	53.7%	0.745	0.615
	AG	45.5%	40.1%	35.0%	39.5%	0.590	0.971
	GG	9.1%	7.7%	0.0%	6.8%		
	A	68.3%	72.2%	82.5%	73.5%	0.468	0.332
	G	31.8%	27.8%	17.5%	26.5%	0.526	0.942

The SF group had over 1.7 times the odds of possessing the *VDR* rs2228570 “risk” TT genotype in comparison to the NSF and TNSF groups (Table 7.2; Table 7.3). Similarly, the SF group had over 1.5 times the odds of possessing the *TNFRSF11A* rs3018362 “risk” AA genotype in comparison to the NSF and TNSF groups and over twice the odds of possessing the *TNFRSF11B* “risk” AA genotype (Table 7.2; Table 7.3).

**Table 7.2:** Odds ratio and confidence interval (CI) statistics for stress fracture injury in SF vs NSF of the 10 investigated SNPs.

<b>SNP</b>	<b>Genetic model</b>	<b>Odds ratio</b>	<b>95% CI</b>
<i>AXIN1</i> rs9921222	T/C	0.96	0.56 – 1.63
	TT/CC	0.93	0.33 – 2.58
	TT/C carriers	1.29	0.55 – 3.05
	T carriers/CC	0.70	0.30 – 1.61
<i>BDNF-AS</i> rs6265	A/G	0.99	0.50 – 1.99
	AA/AG	N/A	N/A
	AA/G carriers	N/A	N/A
	A carriers/GG	1.16	0.52 – 2.55
<i>COL1A1</i> rs1800012	C/A	0.53	0.24 – 1.16
	CC/AA	0.57	0.07 – 4.96
	CC/A carriers	0.71	0.08 – 6.09
	C carriers/AA	0.45	0.18 – 1.11
<i>COMT</i> rs4680	G/A	0.95	0.55 – 1.62
	GG/AA	0.85	0.28 – 2.65
	GG/A carriers	0.76	0.29 – 2.01
	G carriers/AA	1.08	0.46 – 2.53
<i>LRP5</i> rs3736228	A/G	0.98	0.48 – 2.02
	AA/GG	0.86	0.10 – 7.73
	AA/G carriers	0.86	0.09 – 7.58
	A carriers/GG	1.50	0.72 – 3.16
<i>P2X7R</i> rs3751143	C/A	1.03	0.53 – 1.98
	CC/AA	1.14	0.22 – 5.99
	CC/A carriers	1.46	0.28 – 7.59
	C carriers/AA	0.78	0.36 – 1.66
<i>TNFRSF11A</i> rs3018362	A/G	1.03	0.58 – 1.81
	AA/GG	1.58	0.56 – 4.43
	AA/G carriers	2.12	0.79 – 5.67
	A carriers/GG	0.70	0.33 – 1.51
<i>TNFRSF11B</i> rs4355801	A/G	1.33	0.77 – 2.30
	AA/GG	1.36	0.49 – 3.78
	AA/G carriers	2.05	0.95 – 4.46
	A carriers/GG	0.83	0.33 – 2.12
<i>VDR</i> rs2228570	T/C	1.45	0.84 – 2.48
	TT/CC	1.77	0.65 – 4.81
	TT/C carriers	1.89	0.78 – 4.57
	T carriers/CC	1.11	0.51 – 2.41
<i>WNT16</i> rs3801387	A/G	0.83	0.46 – 1.47
	AA/GG	0.74	0.19 – 2.99
	AA/G carriers	0.77	0.36 – 1.64
	A carriers/GG	0.84	0.22 – 3.20

**Table 7.3:** Odds ratio and confidence interval (CI) statistics for stress fracture injury in SF vs TNSF of the 10 investigated SNPs.

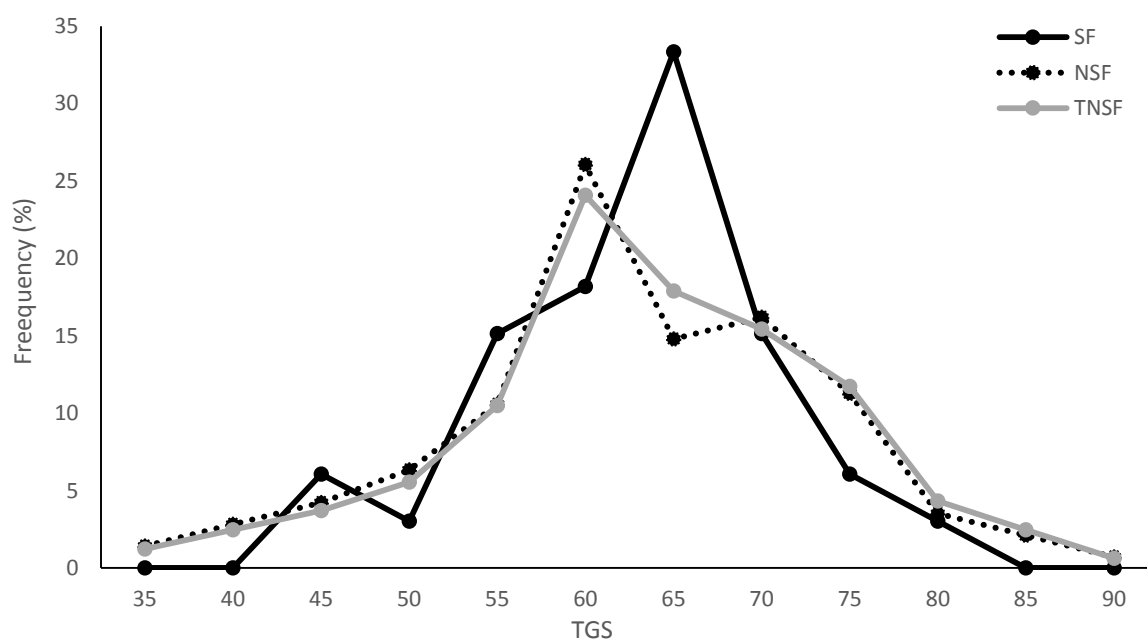
SNP	Genetic model	Odds ratio	95% CI
<i>AXIN1</i> rs9921222	T/C	1.04	0.61 – 1.77
	TT/CC	1.11	0.41 – 3.02
	TT/C carriers	1.36	0.58 – 3.19
	T carriers/CC	0.83	0.37 – 1.89
<i>BDNF-AS</i> rs6265	A/G	0.98	0.49 – 1.94
	AA/GG	N/A	
	AA/G carriers	N/A	
	A carriers/GG	1.14	0.52 – 2.50
<i>COL1A1</i> rs1800012	C/A	0.55	0.25 – 1.21
	CC/AA	0.66	0.08 – 5.73
	CC/A carriers	0.81	0.10 – 6.98
	C carriers/AA	0.47	0.19 – 1.15
<i>COMT</i> rs4680	G/A	1.00	0.59 – 1.71
	GG/AA	0.95	0.31 – 2.92
	GG/A carriers	0.81	0.31 – 2.11
	G carriers/AA	1.19	0.52 – 2.75
<i>LRP5</i> rs3736228	A/G	1.07	0.53 – 2.18
	AA/GG	1.01	0.11 – 9.04
	AA/G carriers	0.98	0.11 – 8.68
	A carriers/GG	1.65	0.79 – 3.43
<i>P2X7R</i> rs3751143	C/A	1.07	0.56 – 2.04
	CC/AA	1.35	0.26 – 7.06
	CC/A carriers	1.68	0.32 – 8.70
	C carriers/AA	0.85	0.40 – 1.81
<i>TNFRSF11A</i> rs3018362	A/G	1.07	0.61 – 1.88
	AA/GG	1.74	0.63 – 4.82
	AA/G carriers	2.30	0.87 – 6.08
	A carriers/GG	0.74	0.35 – 1.56
<i>TNFRSF11B</i> rs4355801	A/G	1.26	0.73 – 2.18
	AA/GG	1.22	0.45 – 3.36
	AA/G carriers	1.92	0.90 – 4.12
	A carriers/GG	0.78	0.31 – 1.96
<i>VDR</i> rs2228570	T/C	1.56	0.92 – 2.65
	TT/CC	2.05	0.77 – 5.51
	TT/C carriers	2.09	0.87 – 5.03
	T carriers/CC	1.22	0.57 – 2.62
<i>WNT16</i> rs3801387	A/G	0.77	0.44 – 1.37
	AA/GG	0.63	0.16 – 2.54
	AA/G carriers	0.72	0.34 – 1.52
	A carriers/GG	0.73	0.19 – 2.77



No differences were observed in TGS between SF vs NSF and SF vs TNSF comparisons ( $P = 0.978$ ;  $P = 0.715$ ) with similar means, modes and medians for TGS observed in all three cohorts (Table 7.4). ROC analysis determined that TGS frequency distribution could not distinguish SF from NSF (AUC = 0.498,  $P = 0.977$ ; Figure 7.3) or SF from TNSF runners (AUC = 0.479,  $P = 0.703$ ; Figure 7.3).

**Table 7.4:** Mean (SD), mode and median TGS of SF, NSF and TNSF groups.

Group	TGS Mean (SD)	Mode	Median	Comparisons and P-value
SF (n = 33)	62.73 (8.01)	65.00	65.00	SF vs NSF 0.978
NSF (n = 142)	62.78 (10.51)	60.00	60.00	
TNSF (n = 162)	63.43 (10.37)	60.00	65.00	SF vs TNSF 0.715



**Figure 7.3:** TGS frequency distribution in SF, NSF and TNSF groups.

## 7.4 Discussion

The aim of this chapter was to investigate the influence of BMD-associated genetic variants on stress fracture occurrence in high-level endurance runners. No differences in genotype or allele frequency were observed for any SNP between SF vs NSF and SF vs TNSF comparisons but the “risk” (lower BMD-associated) *TNFRSF11A* rs3018362 and *TNFRSF11B* rs4355801 AA genotypes were more frequent in the SF group before multiple testing correction. Additionally, the SF group had over 1.5 times and almost twice the odds of possessing the *TNFRSF11A* rs3018362 and *TNFRSF11B* rs4355801 AA genotype, respectively, in comparison to the NSF and TNSF groups.

Although no associations were present after multiple testing correction, runners who had suffered a stress fracture (SF) possessed >9% higher frequency of the “risk” lower BMD-associated *TNFRSF11A* rs3018362 AA genotype than runners who had no history of stress fracture injury confirmed via scan (NSF and TNSF). There was also a ~17% higher frequency of the “risk” lower BMD-associated *TNFRSF11B* rs4355801 AA genotypes in the SF than NSF runners in the recessive analysis model. *TNFRSF11A* and *TNFRSF11B* encode RANK and OPG respectively, forming part of the RANK/RANKL/OPG signalling pathway, which plays an essential role in regulating osteoclast differentiation and activity following mechanical loading (Boyle et al., 2003) and is therefore, important for bone turnover.

Although not directly causing the disease, the AA genotype of the *TNFRSF11A* rs3018362 SNP has been associated with Paget’s disease of bone and reduced BMD (Styrkarsdottir et al., 2009; Albagha et al., 2010) as well as stress fracture in elite athletes before multiple testing correction (Varley et al., 2015). Paget’s disease is characterised by abnormal bone architecture via irregular bone remodelling (extensive

osteoclast-mediated bone resorption before defective osteoblast-mediated bone repair), causing bone expansion and softening, resulting in overproduction of poor quality bone (Delmas and Meunier, 1997; Whyte, 2006). Although the direct mechanism underpinning how the AA genotype may affect the protein product and subsequently influence BMD, it could be hypothesised that possessing the AA genotype aids in increasing bone turnover, raising the amount of poor quality bone and consequently increasing risk of stress fracture in athletes.

Previous study has suggested the A allele to be associated with reduced BMD and *TNFRSF11B* expression, as well as increased risk of osteoporotic fracture via GWAS (Richards et al., 2008). Consequently, this reduced expression and associated lower BMD via possession of the A allele may explain why runners with stress fracture injury history had increased odds of possessing the A allele. Despite these findings, Varley et al. (2015) observed the G allele, rather than the A allele, to be associated with multiple stress fracture injury history in the Stress Fracture Elite Athlete (SFEA) cohort. BMD was not measured in the Varley et al. (2015) investigation and thus other stress fracture risk factors such as training load, body composition and type of mechanical loading (as discussed throughout the thesis) may have provided a greater contribution to stress fracture incidence than genetics in that investigation. The underpinning mechanisms of how genetic variation in SNPs in the RANK/RANKL/OPG pathway such as the ones investigated in this chapter may be influencing BMD (and subsequently stress fracture) is not well understood as discussed in Chapter 5. Alongside influencing osteoclastogenesis, a *TNFSF11* (RANKL) SNP (rs9594738) was found to be associated with BMD and the region where harboured appeared to be stimulated by the presence of vitamin D, indicating a potential role in the RANK/RANKL/OPG equilibrium (Yoskovitz et al., 2013).

Runners with previous stress fracture history had over 1.7 and 2.0 times the odds of possessing the *VDR* rs2228570 “risk” TT genotype in comparison to those who had no history of stress fracture confirmed via scan (NSF and TNSF). This observation is in agreement with a previous study in athletic populations, where the *VDR* rs2228570 T allele was associated with stress fracture in the Stress Fracture Elite Athlete Cohort (SFEA) (Varley et al., 2017) as well as in military recruits (Chatzipapas et al., 2009). A number of *VDR* SNPs have been proposed to influence BMD and it has proposed that genotype variation can regulate *VDR* concentration, which could potentially impact parathyroid hormone or vitamin D binding and as a consequence, calcium absorption (McClung and Karl, 2010). The *VDR* rs2228570 C allele appears to increase transactivation (protein expression) compared to the T allele - this increased biological activity (and associated increased intestinal absorption of calcium) could explain why higher BMD has been reported in those with the CC genotype (Arai et al., 1997; Colin et al., 2000; Uitterlinden et al., 2004; Ames et al., 1999). Consequently, this enhanced BMD through C allele possession and the associated reduced risk of stress fracture may explain why runners with no history of stress fracture were 1.5 times less likely to carry the T allele in this chapter and why the T allele has been associated with stress fracture in previous investigation.

A number of risk factors (alongside the aforementioned ones above) such as dietary intake, hormonal levels and biomechanical gait have been proposed as determinants of stress fracture in endurance runners, which may explain why no genetic associations with stress fracture were observed after multiple testing correction in this chapter. It could also be hypothesised that BMD may not be involved in the direct pathophysiology of stress fracture injury whilst pathophysiological differences between osteoporotic and stress fracture exist. Ultimately, other bone phenotypes may have a

greater impact on risk, such as lower cortical area and bone size, ultimately influencing bone strength (Popp et al., 2009; Evans et al., 2008).

Further research, however, is needed to support the evidence of these risk factors as meta-analysis has indicated that previous stress fracture history and female sex are the only two factors that have strong evidence to support an association with stress fracture in endurance runners (Wright et al., 2015). Although no association for any SNP with stress fracture was observed in this chapter, this could suggest that these other factors are contributing to stress fracture incidence more so than genetics in athletic populations. However, previous investigations reported genetic associations with stress fractures in athletic populations (Varley et al., 2015; Varley et al., 2016; Varley et al., 2017) and, although it was not significant after multiple testing correction, *TNFRSF11A* rs3018362 was associated with stress fracture injury in both this chapter as well as the runners of the SFEA cohort (Varley et al., 2015).

Differences in the findings between this chapter and the only other studies to date investigating genetic associations of stress fracture in athletes via the SFEA, however, do exist (Varley et al., 2015; Varley et al., 2016; Varley et al., 2017). *LRP5* rs3736228, *COL1A1* rs1800012 and *WNT16* rs3801387 observed no association with stress fracture in the SFEA cohort as in agreement with this chapter (Varley et al., 2017). *TNFRSF11A* rs3018362 was associated with stress fracture in this chapter and with the SFEA cohort before multiple testing correction (Varley et al., 2015), whilst *P2X7R* 3751143 was associated with stress fracture injury only in the SFEA cohort (Varley et al., 2016; Varley et al., 2017). These differences between studies may relate to differences in sample size and participant characteristics. Whilst the SFEA cohort (Varley et al., 2015; Varley et al., 2016; Varley et al., 2017) comprised a larger sample size of 125 athletes who had suffered stress fractures in comparison to the current chapter, these comprised athletes of mixed abilities across of a number of sports

including football, rowing and boxing, where substantial variation in athlete physical characteristics exist. The athlete sample in this chapter, however, consisted wholly endurance runners of similar standards who have congruent physical characteristics. Specifically, the SF male runners from this chapter were  $1.80 \pm 0.06$  m and  $70.6 \pm 5.0$  kg in height and body mass whilst the NSF runners were  $1.78 \pm 0.06$  m and  $66.4 \pm 6.8$  kg. In the SFEA, male athletes who had suffered stress fractures were  $1.85 \pm 0.07$  m and  $82.9 \pm 10.6$  kg and those who had no stress fracture history were  $1.82 \pm 0.07$  m and  $79.6 \pm 9.4$  kg in height and body mass, respectively. Alongside these differences in physical characteristics, variability in stress fracture location for different sports is also likely and thus, these different locations may be comprised of varying amounts of trabecular and cortical bone. It has been proposed that different genes and phenotypes may have specific roles in cortical or trabecular bone adaptation as outlined in Chapter 5. Utilising a more homogenous cohort can somewhat strengthen the statistical power and better assess the genetic influence on stress fracture by reducing the variability in these factors.

The collective genetic association, as part of a TGS, found no association with stress fracture in this chapter. A large number of genes have been proposed to be associated with BMD and osteoporotic fracture in non-athlete populations (Hsu and Kiel, 2012). It is possible that certain variants may have a greater influence in athletes than non-athlete populations, which may explain why genes associated with non-athlete and untrained populations have not been replicated in this chapter or other studies in athletes (Varley et al., 2018; Varley et al., 2015; Varley et al., 2016; Varley et al., 2017). This variation association may be dependent upon the particular biological function of that variant. Specifically in relation to endurance runners, certain genes/SNPs may be important in the adaptation of bone to large volumes of mechanical loading and thus may demonstrate a greater influence on BMD and/or stress fracture risk than in non-

endurance or non-athletic participants. For example, *VDR* and *TNFRSF11A* have been suggested to influence bone through a mechanically induced interaction and consequently associated with bone phenotypes in athletic populations, although the specific mechanisms are not yet understood. *COL1A1* rs1800012 was not associated with stress fracture in this chapter or in the elite athlete cohort (Varley et al., 2017), although it has been associated with degenerative bone microarchitecture (Mann et al., 2001; Grant et al., 1996; Dytfeld et al., 2016) and associated with BMD (Jin et al., 2011). It could therefore be hypothesised that *COL1A1* rs1800012 may have greater influence on other conditions or populations, such as osteoporosis imperfecta (Marini et al., 2007), where the mechanical loading influence will be less prominent. These potential function-specific differences may explain why some variants and not others are associated with stress fracture in athletic populations. Moreover, this becomes more complex when considering that pleiotropic effects between BMD and lean mass (a shared genetic component of 43%) have been recently reported (Medina-Gomez et al., 2017). Thus, variability in the muscle capacity to absorb bone could impact the bone tissue and subsequent fracture risk (Brotto and Bonewald, 2015; Varley et al., 2018). It is also underdetermined how gene-gene interactions or SNPs that are involved within the same signalling pathway may interact to impact BMD. Overall, stress fracture injury is likely to be polygenic in nature but is also influenced by environmental determinants such as dietary intake and training characteristics (Wentz et al., 2012).

This chapter suggests that *AXIN1* 9921222, *BDNF-AS* rs6265, *COL1A1* rs1800012, *COMT* rs4680, *LRP5* rs3736228, *P2X7R* rs3751143, *TNFRSF11A* rs3018362, *TNFRSF11B* rs4355801, *VDR* rs2228570 and *WNT16* rs3801387 are not associated with stress fracture incidence in high-level endurance runners. However, associations with the *TNFRSF11A* rs3018362 and *TNFRSF11B* rs4355801 (lower BMD-

associated) AA genotypes to be more frequent in runners who had suffered a stress fracture in comparison to those who had no stress fracture injury history before multiple testing correction were observed. Additionally, runners who had previous stress fracture history had over 1.5 times the odds of possessing the *TNFRSF11A* rs3018362 or *TNFRSF11B* rs4355801 “risk” AA genotypes or the *VDR* rs2228570 “risk” TT genotype in comparison to runners with no stress fracture injury history. This data, alongside previously reported functional studies and associations with stress fracture as well as BMD, could suggest that variation in the *TNFRSF11A* rs3018362, *TNFRSF11B* rs4355801 and *VDR* rs2228570 SNPs may influence stress fracture occurrence. Further study, utilising larger sample sizes of homogenous cohorts to increase statistical power is needed to provide greater evidence of these potential genetic associations with stress fractures in athletes.



# **Chapter 8:**

# **Discussion**

## **8.1 Overview:**

The overall aim of the current thesis was to investigate the genetic associations with BMD, stress fracture incidence and performance in high-level endurance runners and compare these to a non-athlete cohort to explore genotype-physical activity interactions.

### **8.1 Bone mineral density**

#### *8.1.1 Bone mineral density in high-level endurance runners and non-athletes*

Female runners possessed ~4% higher LBMD but similar TBMD and LSBMD than their non-athlete counterparts, whilst no differences in total-body T-score or Z-score were present between the two cohorts. Higher site-specific BMD (i.e. LBMD) in endurance runners has also been observed in previous investigation (Duncan et al., 2002; Scofield and Hecht, 2012; Nevill et al., 2003; Brahm et al., 1997) and these findings highlight the site-specific nature of bone adaption following mechanical loading. Contradictory findings, reporting lower or low in female endurance runners, specifically at sites of smaller magnitudes of loading, however, have been reported (Pollock et al., 2010; Barrack et al., 2008b). This may be due to reduced energy availability or the specific training programme completed by the individual runner as discussed in Chapter 3. It must be noted that the only other study investigating BMD in a homogenous group of UK high-level female endurance runners observed low BMD (Z-score of -1.0 to -2.0) in some of the runners investigated (Pollock et al., 2010). The findings from this investigation, therefore, have to be interpreted with caution and further research is needed to confirm the nature of BMD in female high-level endurance runners.

Male endurance runners possessed lower (~10%) LSBMD but similar TBMD and LBMD in comparison to the male non-athletes. Total-body T-score and Z-score comparisons between the two groups also revealed no differences. Lower LSBMD in male endurance runners has been reported previously (Hind et al., 2006), which suggests some male runners may be at risk of reduced energy availability, which has been highlighted in the recent IOC consensus statement (Mountjoy et al., 2018). Consequently, the combination/relationship of energy availability, low body mass and large training volumes, as well as the difference in BMD in comparison to the non-athletes occurring at a site that is subjected to less loading, may explain why lower LSBMD was observed in the male runners in comparison to non-athletes.

These findings from Chapter 3 suggest that male runners have lower BMD at sites of less loading in comparison to non-athletes, and could therefore be at risk for complications associated with low BMD such as fracture or osteoporosis. Large variance in BMD was present in both the runners and non-athletes for both males and females, which highlights the substantial genetic contribution to BMD, even when physical activity/mechanical loading level is relatively homogenous as is the case for the endurance runners.

#### *8.1.2 Bone mineral density and stress fracture occurrence in high-level endurance runners*

Chapter 6 revealed no differences in any bone phenotype between the female runners who had suffered a stress fracture (SF) in comparison to those who had no stress fracture injury history (NSF). However, lower TBMD, T-score and Z-score were observed in SF female runners in comparison to those who had reported stress fracture symptoms but had a negative scan result or no scan diagnosis (SSF). The higher BMD observed in the SSF than SF female runners could indicate that the SSF

runners did not suffer a true stress fracture (visible cortical fracture) due to possessing higher BMD as discussed in Chapter 6. Previous studies have produced inconsistent findings in regards to BMD association with stress fracture (Crossley et al., 1999; Duckham et al., 2012; Bennell et al., 1996; Kelsey et al., 2007). Possessing lower BMD can contribute to stress fracture incidence through reducing bone strength, resulting in increased accumulation of microdamage via repetitive loading over a prolonged period (Bennell et al., 1999). Stress fracture occurrence, however, is a multi-factorial phenotype, and other factors such as biomechanical gait, other bone characteristics and genetics have all been associated with stress fracture injury, which may explain the inconsistent literature.

In contrast to females, male runners who had suffered at least one stress fracture possessed higher TBMD, LBMD, T-score and Z-score than non-stress fracture runners. Analysing Chapters 3 and 6 collectively alongside the functional mechanism behind stress fracture and lower BMD, it would be expected that BMD would be lower in those who had suffered a stress fracture. However, previous study investigating BMD and stress fracture occurrence in male endurance runners has primarily suggested that BMD is not associated with stress fracture incidence (Crossley et al., 1999). Consequently, other risk factors, rather than BMD, as mentioned previously, may provide a more pivotal role in stress fracture occurrence in male endurance runners. These findings, however, have to be interpreted with caution as only seven male endurance runners formed the stress fracture group and therefore, further study is still required to provide confidence to this conclusion.

## 8.2 Genetic associations

### 8.2.1 *WNT16* rs3801387

In Chapter 3, elite runners possessed a lower frequency of AA genotype than the non-athletes and sub-elite runners. This suggests that possessing the AA genotype is beneficial for reaching elite status through protection against stress fracture injury and potential training interruption but does not influence initial capacity to reach high-level marathon performance. When *WNT16* rs3801387 was explored in relation to BMD in the endurance runners in Chapter 5, runners who were AA genotype possessed lower LSBMD and Z-score, and a tendency to possess LBMD than non-athletes with AA genotype. Additionally, a tendency for runners who were AG genotype to possess higher LBMD than non-athletes with AG genotype was observed, suggesting a differing association between genotype and BMD for the two cohorts. The findings from Chapters 3 and 5 (combined with previous research reporting the A allele association with lower BMD) could therefore indicate that a lower frequency of *WNT16* rs3801387 AA genotypes exists in elite endurance runners due to associated lower BMD that could ultimately influence ability to train and thus performance. Higher BMD in the AG genotype runners than non-athletes could also indicate that the G allele is important for BMD in male endurance runners via a mechanical loading interaction and a subsequent influence on the Wnt signalling pathway. *WNT16*-mechanical loading interaction effects for bone phenotypes have been reported in both mice and humans (Wergedal et al., 2015; Mitchell et al., 2016) providing support for this notion. These interesting findings regarding *WNT16* rs3801387, however, need to be taken with caution, as no *WNT16* rs3801387 association with stress fracture occurrence was observed in a previous study (Varley et al., 2018) or Chapter 7.

### 8.2.2 *P2RX7* rs3751143

The *P2RX7* rs3751143 was associated with both high-level endurance runner status in Chapter 3 and with BMD in females in Chapter 5 but no association with stress fracture was observed in Chapter 7. In Chapter 3, surprisingly, the “risk” (lower BMD-associated) C allele was more frequent in the runners than controls. It could be proposed that the C allele serves another beneficial purpose via the cardiovascular system (through the release of pro-inflammatory mediators and cell proliferation) to aid in reaching high-level marathon performance, as discussed in Chapter 3.

In chapter 5, *P2RX7* rs3751143 AA genotypes possessed higher TBMD, LBMD, T-score and Z-score than AC genotypes (as well as C allele carriers when investigated in the dominant analysis model) in the total female cohort, which is agreement with previous studies (Wesselius et al., 2013). Tendencies for AA genotype runners to possess higher than AC genotypes but CC to possess higher than AC genotypes was also observed in the female runners. It therefore could be that a potential CC genotype-mechanical loading interaction exists but this was not apparent in Chapter 5 and no effect of *P2RX7* rs3751143 on bone phenotypes was reported following a 12-week training programme in academy football players (Varley et al., 2018). The findings could also indicate that runners may be able to combat a potential disadvantageous genetic predisposition and enhance BMD through mechanical loading as has been reported previously in children (Mitchell et al., 2016), which may explain why no association with stress fracture was present in Chapter 7. Additionally, *P2RX7* SNPs have been associated with cortical thickness and CSA (Varley et al., 2018) and thus may influence stress fracture incidence through other bone phenotypes, rather than BMD. Overall, the AA genotype appears to be associated with BMD females but does not seem to affect athlete status or influence stress fracture in high-level endurance runners.

### 8.2.3 *COL1A1 rs1800012*

In males, *COL1A1* rs1800012 AA genotypes possessed higher LBMD than AC genotypes whilst AA genotypes also possessed higher TBMD and LBMD than C allele carriers (in the dominant analysis model). This association has been primarily reported in females (Jin et al., 2011; Ralston et al., 2006), which may be due to a lack of research in male populations. No further significant associations were present in Chapter 5 or within any other chapter, which would suggest that *COL1A1* rs1800012 does not appear to be associated with BMD or stress fracture in endurance runners as has also been observed previously (Varley et al., 2017) and is not influenced by physical activity. Consequently, further research regarding *COL1A1* rs1800012 is still warranted to explore potential mechanisms and determine if *COL1A1* rs1800012 genotype differences in BMD are population-specific.

### 8.2.4 *COMT rs4680*

In this thesis, *COMT* rs4680 was not associated with any particular bone phenotype or stress fracture but appeared to influence endurance runner status in Chapter 3. Specifically, the elite runners possessed a much lower frequency of the “risk” (lower BMD-associated) GG genotype in comparison to the non-athletes. This could suggest that elite endurance runners are genetically predisposed to be at less risk of lower BMD by possessing at least one “protective” A allele. Furthermore, GA genotypes were overrepresented in athletes compared to non-athletes. Although, a *COMT* rs4680 genotype-cohort interaction was not observed in Chapter 5, GA genotype runners possessed the highest TBMD, LBMD and LSBMD in comparison to GG or AA genotypes. Therefore, a larger sample size may increase statistical power and provide further evidence for the aforementioned hypothesis.

### 8.2.5 *TNFRSF11A* rs3018362

*TNFRSF11A* rs3018362 was associated with TBMD in males in Chapter 5 as well as stress fracture in Chapter 7 before multiple testing correction. In Chapter 5, higher TBMD was observed in the GG genotypes compared to GA (as well as compared to A allele carriers in dominant analysis model) genotypes in both the total male cohort and non-athlete analyses. These findings could suggest that possessing *TNFRSF11A* rs3018362 GG genotype may be important for BMD in non-athlete populations, as suggested by GWAS and candidate gene association study (Paternoster et al., 2010; Styrkarsdottir et al., 2009). No genotype-dependent differences in TBMD were observed in the runner analysis, which could indicate that runners may be able to compensate for a potential disadvantageous genetic predisposition through completion of large volumes of mechanical loading and subsequently increasing BMD. This potential protective mechanism has been observed in children, albeit in different variants (Mitchell et al., 2016).

*TNFRSF11A* rs3018362 was associated with stress fracture in Chapter 7 before multiple testing correction and is therefore in agreement with previous study (Varley et al., 2015). Runners who had suffered a stress fracture had over 1.5 times the odds of possessing the *TNFRSF11A* rs3018362 AA genotype in comparison to those who had no stress fracture injury history. *TNFRSF11A* rs3018362, however, was not associated with BMD in the runners in Chapter 5. This could indicate that a genotype-dependent disruption within a particular phase of the remodelling process following mechanical loading, rather than a lower BMD, could aid in aetiology of stress fracture incidence in endurance runners. Overall, it is surprising to have not observed more associations with *TNFRSF11A* rs3018362 throughout the thesis, given the functional influence on bone and the previous associations with BMD (Paternoster et al., 2010; Styrkarsdottir



et al., 2009) although the variant may mediate stress fracture incidence in endurance runners.

#### 8.2.6 *BDNF-AS rs6265*

The results from Chapter 5 showed a *BDNF-AS rs6265*-cohort interaction in the female analysis, with runners who were GG (higher BMD-associated) genotype found to possess lower LSBMD than non-athletes of the same genotype. It is difficult to elucidate why the GG genotype may be disadvantageous for LSBMD in endurance runners (due to lack of research on this SNP to date) but it is interesting to note that lower LSBMD has been reported in female endurance runners previously, although not in this investigation. Overall, *BDNF-AS* association with BMD has not been explored in athletic cohorts and has had relatively little investigation in human populations. To the author's knowledge, this is only the second study to have assessed *BDNF-AS* influence on BMD in humans, and therefore, additional study is still warranted in both candidate-gene association and functional studies to provide evidence for a potential mechanism and outcome for BMD.

#### 8.2.7 *VDR rs2228570, TNFRSF11B rs4355801 and AXIN1 rs9921222*

*VDR rs2228570* has been associated with BMD in a number of populations, as well as shown a gene-physical activity interaction and association with stress fracture in athletes (Nakamura et al., 2002b; Nakamura et al., 2002a; Varley et al., 2017). *TNFRSF11B rs4355801* and *AXIN1 rs9921222* have also been associated with BMD (Estrada et al., 2012) and are involved in bone metabolism pathways. Runners with previous stress fracture history had approximately 1.5 times and twice the odds of possessing the *VDR rs2228570* “risk” T allele and *TNFRSF11B rs4355801* “risk” AA genotype in comparison to runners with no stress fracture injury history, respectively. In addition, the *AXIN1 rs9921222* “risk” (lower BMD-associated) T allele and TT

genotype were overrepresented in the runners (particularly the more elite) compared to the non-athletes before multiple testing correction.

None of these observations, however reached significance and therefore, are not in agreement with the aforementioned previous studies. Differences in the findings could be due to the influence of other genes and their associated proteins in their particular signalling pathways as discussed in Chapter 5. Additionally, age-associated effects could also determine potential genetic associations, for example, in the case of *VDR*, Riggs et al. (1995) proposed that *VDR* influence on BMD has no effect after the age of 70, with differences in BMD only allele-dependent in the pre- and not the post-menopausal women. Moreover, variability in the sports and the physical attributes of the athletes in SFEA cohort, combined with the differences in mechanical loading characteristics may also contribute to differences in results.

#### 8.2.8 *LRP5* rs3736228

No significant findings for *LRP5* rs3736228 were observed for any chapter of this thesis. It is surprising to observe no associations with *LRP5* rs3736228 given the functional effect of *LRP5* in bone metabolism and findings from previous studies (Robinson et al., 2006; Krishnan et al., 2006; Kiel et al., 2007). No association with *LRP5* rs3736228 and stress fracture in athletes has also been observed previously (Varley et al., 2017). Prior studies investigating *LRP5* and BMD have primarily been conducted in older adults, so it could be hypothesised that *LRP5* variants are more important for BMD in older people.

#### 8.2.9 Total genotype score

Polygenic profiling was conducted to ascertain the combined influence of the 10 investigated SNPs on athlete status, marathon performance, BMD and stress fracture

incidence but no significant findings were observed throughout the thesis. Polygenic analysis to examine the potential combined genetic influence on BMD or osteoporotic fracture risk has produced exciting outcomes in regards to classifying fracture risk (Lee et al., 2014). Only one study so far has completed such analysis in athletic populations but initial studies have shown a positive outcome in regards to injury in a small number of triathletes (Goodlin et al., 2015). As outlined in Chapter 3, utilising a TGS model requires allocating a score based on existing literature and therefore, each SNP homozygote associated with higher BMD is given a score of 2, heterozygotes scoring 1 and the other homozygote given 0, deemed the “risk” genotype. Incorporating this approach allows analysis of potential genetic associations in a polygenic, rather than singular nature, which can provide a better assessment of a genetic contribution to a particular phenotype and is also beneficial given the difficulty of calculating the effect size of single or multiple SNPs. Polygenic profiling, however is limited by this method of assigning the individual contribution of each SNP as it is extremely likely that the contribution of each genotype for each SNP to BMD is variable. Overall, utilising an individual SNP approach allows initial assessment of specific associations with a phenotype of interest, such as BMD, in comparison to using a TGS method. This will provide more convincing evidence regarding the individual variants in question to help develop polygenic profiling approaches and allow for more useful estimations of the polygenic influence on athlete status, BMD and stress fracture risk in endurance runners.

### **8.3 Conclusion**

This current thesis investigated 10 BMD-associated genetic variants in a novel population (high-level endurance runners) in relation to athlete status, performance, BMD and stress fracture incidence. Consequently, this thesis has extended the growing body of research suggesting that differences in BMD exist between endurance

runners and non-athletes, whilst BMD variability may also influence stress fracture incidence. Moreover, there appears to be a genetic influence on BMD and stress fracture that may occur via a gene-mechanical loading interaction, whilst BMD-associated variants may also influence high-level endurance runner status but not marathon performance time directly. Athlete status, BMD and stress fracture susceptibility, however, are all likely to be highly polygenic as well as determined by environmental factors as discussed throughout this thesis.

## **8.4 Limitations**

The strength of this thesis was the large homogenous cohort of high-level endurance runners that represent one of the largest samples studied to date for genetic associations as well as exploration of BMD and stress fracture in such a population. Furthermore, it is the first study to investigate the genetic association of BMD-associated variants with athlete status, performance, BMD and stress fracture incidence in a large homogenous high-level endurance runner cohort. Although these points represented strengths of the thesis, it is difficult to recruit large numbers of a particular population such as high-level endurance runners that meet the criteria detailed in Chapter 2. Genetic association studies require large sample sizes to ascertain the individual variant effect on a phenotype. However, due to the problems associated with measuring physical activity and mechanical loading as discussed in Chapter 1, utilising a relatively homogenous population that completes large volumes of mechanical loading to examine any potential gene-mechanical loading interaction remains a potentially informative approach.

## 8.5 Directions for future research

Firstly, male runners may possess lower BMD than non-athletes at sites of less loading (i.e. lumbar spine), whilst female runners possess higher site-specific BMD (i.e. leg) than their non-athlete counterparts. These findings suggest that male runners may be at risk for low BMD at the lumbar spine as suggested previously (Hind et al., 2006). Despite these associations in Chapter 3, BMD appeared to only influence stress fracture incidence in female endurance runners and not males in Chapter 6, suggesting that stress fracture is a multi-factorial phenotype influenced by biomechanical gait as well as physical and training characteristics. Further research in large homogenous cohorts of endurance runners is needed to confirm these findings so that preventative strategies can be implemented to avoid potential skeletal health issues and stress fracture incidence. Alongside differences in BMD between the runners and non-athletes as well as stress fracture groups, a huge variability in BMD was observed, highlighting the large genetic component to BMD.

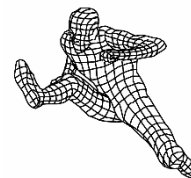
Genetic analysis revealed the *WNT16* rs3801387 and *BDNF-AS* rs6265 to be associated with BMD via a gene-cohort interaction, whilst *WNT16* rs3801387 was also associated with endurance runner status. Consequently, further research investigating *WNT16* rs3801387 and *BDNF-AS* rs6265 in relation to other athletic populations is warranted to elucidate the exact contribution of *WNT16* rs3801387 to these specific phenotypes. *P2RX7* rs3751143 and *COL1A1* rs1800012 were associated with BMD in females and males respectively, providing further evidence for their influence on bone, whilst *P2RX7* rs3751143 was also associated with athlete status, although this may be via another biological mechanism rather than enhanced BMD. It would be interesting to continue additional research of these two variants across different populations to determine if there are particular time points across the human lifespan or specific cohorts where they may be particularly influential on BMD. *COMT* rs4680

was associated with endurance runner status in Chapter 3 and has demonstrated potential gene-physical activity interactions previously, and thus, further study in athletic populations is suggested. It would be particularly interesting to understand if the type of loading that is of greater magnitude (higher peak force/strain rate/multidirectional), as seen in sports other than endurance running, may affect potential gene-mechanical loading interactions. No SNPs were associated with stress fracture, although both *VDR* rs2228570 and *TNFRSF11B* rs4355801 demonstrated increased odds ratios when investigating genetic association with stress fracture. Both these variants have been associated with stress fracture in athletic populations previously. Functional studies that can determine the possible genetic pathogenesis with stress fracture incidence would, therefore, be a valuable future research focus. Although age, running distance volume (and menstrual status) were collected as part of this investigation, these were only incorporated to provide descriptive and training characteristics of the population. Menstrual status influence on BMD was explored in Chapter 1 and no differences were observed between those who were deemed eumenorrheic in comparison to those who were termed as amenorrheic. Additionally, diluting the groups to smaller sample sizes based on age, running distance volume or menstrual status to assess genetic associations would potentially reduce the confidence or power of these potential associations. In future, it would be interesting to use this type of information (training characteristics, age and menstrual status) to explore any potential genetic associations when larger sample sizes have been gathered. Such action would allow further exploration into the importance of genetics in comparison to menstrual cycle, for example. Additionally, specific research routes of interest could include whether certain variants are important during particular periods of BMD accrual or loss, or for sports with particular mechanical loading patterns.

# **Appendices**

## **Appendix 1:**

## Department of Exercise and Sport Science



### Informed Consent Form

**(Both the investigator and  
participant should retain a copy of this form)**

Name of Participant:

Principal Investigator: Dr Alun Williams

Project Title: The Genetic Profile of Elite Athletes

Ethics Committee Approval Number: 12.07.11 (i)

### Participant Statement

I have read the participant information sheet for this study and understand what is involved in taking part. Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without giving a reason. Any concerns I have raised regarding this study have been answered and I understand that any further concerns that arise during the time of the study will be addressed by the investigator. I therefore agree to participate in the study.

It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the The University Secretary and Clerk to the Board of Governors, Manchester Metropolitan University, Ormond Building, Manchester, M15 6BX. Tel: 0161 247 3400 who will undertake to investigate my complaint.

Signed (Participant)

Date

Signed (Investigator)

Date



# The Genetic Profile of Elite Athletes: Questionnaire

Thank you for your interest in our research study. Please answer the following questions about your ethnic origin, athlete status, and your training, diet and injury history.

## SECTION A: Questions concerning your ethnic background.

Participant ID code: \_\_\_\_\_ Date of birth: \_\_\_\_\_

Gender (please tick): Male ☐ / Female ☐ Height (in metres): \_\_\_\_\_

Nationality (as on passport, e.g. British): \_\_\_\_\_ Body weight (in kg): \_\_\_\_\_

What is your ethnic group? Please tick the appropriate box.

**A) White:** English ☐ Scottish ☐ Welsh ☐ N. Irish ☐ Irish ☐  
French ☐ South African ☐ New Zealander ☐ Australian ☐ Other ☐

If other, please state here: \_\_\_\_\_

**B) Mixed:** White & Black British ☐ White & Black Caribbean ☐ White & Black African ☐ White & Asian ☐ White & Latin American ☐ Other ☐

If other, please state here: \_\_\_\_\_

**C) Asian:** British ☐ Indian ☐ Pakistani ☐ Chinese ☐ Japanese ☐ Other ☐

If other, please state here: \_\_\_\_\_

**D) Black:** British ☐ Caribbean ☐ African ☐ Other ☐

If other, please state here: \_\_\_\_\_

**E) Latin American:** Brazilian ☐ Argentinian ☐ Mexican ☐ Colombian ☐ Other ☐

If other, please state here: \_\_\_\_\_

**F) Pacific Islands:** Samoa ☐ Fiji ☐ Tonga ☐ PNG ☐ Other ☐

If other, please state here: \_\_\_\_\_

**G) Other ethnic background:** ☐ Please state here: \_\_\_\_\_

I do not wish to state my ethnic origin ☐

Using the ethnic groups above as a guide, please tell us the ethnic origin of your:

Mother: \_\_\_\_\_ Don't know: ☐

Father: \_\_\_\_\_ Don't know: ☐

Mother's mother: \_\_\_\_\_ Don't know: ☐

Mother's father: \_\_\_\_\_

Don't know: ☐

Father's mother: \_\_\_\_\_

Don't know: ☐

Father's father: \_\_\_\_\_

Don't know: ☐

### **Blood donation**

We would like to take a small (5 mL) blood sample from a vein in your arm. Before doing so, please answer the following safety questions.

1. Have you ever been infected with a blood-borne disease? \_\_\_\_\_ Yes ☐ No ☐
2. Are you anaemic or receiving treatment for anaemia or iron deficiency? \_\_ Yes ☐ No ☐

If you have answered YES to any of these questions and/or you would prefer not to provide a blood sample, a saliva sample may be provided instead.

**PLEASE TURN OVER**

**SECTION B: Questions concerning your athlete status.**

1. What is/was your main playing sporting discipline/event?

---

---

2. Please state which event(s) you participate(d) in and your preferred event?

---

---

---

3. What is your official PB(s) for these events?

---

---

---

4. Please state the highest level that you have competed: i.e. international (represented your country); national (represented your region/county/club in a national league/competition); regional (represented your club/town/university in a local/area league/competition)?

---

---

---

Please state the name of the highest standard league/competition in which you have competed, your placing in that competition and the date(s) you competed at that level:

---

---

---

5. Have you any other athletic achievements? If so please state highest achievements and include relevant details:

---

---

**SECTION C: Questions concerning your training.**

1. Typically, how many hours do you train a week?

---

2. Typically, what is your average running distance per week?

---

**PLEASE TURN OVER**

**SECTION D: Questions concerning your injury history.**

1. Ignore this, please move to question 2.

2. Have you ever fractured a bone? Yes ☐ No ☐

3. If Yes, please give details of the bone(s) you broke and at what age you broke them.

Bone	Age
<i>e.g. upper leg/femur</i>	<i>e.g. 20</i>

4. Have you ever been told that you have had a STRESS FRACTURE (or micro-fracture) injury? Yes ☐ No ☐

5. If Yes, please give details of the bone/bones where the stress fracture occurred and at what age the fracture occurred.

Bone	Age
<i>e.g. shin/tibia</i>	<i>e.g. 20</i>

6. If Yes, was it confirmed by a bone scan, e.g. MRI, X-ray, CT scan? Yes ☐ No ☐

7. Have you ever suffered from prolonged shin pain during exercise that does not go away for weeks? Yes ☐ No ☐

8. Does anyone in your close family suffer from OSTEOPOROSIS or FRAGILE bones? Yes ☐ No ☐ Don't know ☐
9. Has anyone in your close family ever had a STRESS FRACTURE? Yes ☐ No ☐ Don't know ☐

**PLEASE TURN OVER**

10. Have you ever ruptured your tendon? Yes ☐ If yes, which tendon? No ☐  
*e.g. Achilles*

11. If Yes, please give details of how this occurred and at what age.

<b>Activity</b>	<b>Age</b>
<i>e.g. sprinting</i>	<i>e.g. 20</i>

12. Have you ever suffered from prolonged tendon pain during exercise that does not go away for weeks? Yes ☐ If yes, which tendon? No ☐  
*e.g. Achilles*

13. Have you ever been told that you have had tendonitis? Yes ☐ If yes, which tendon? No ☐  
*e.g. Achilles*

14. If Yes, was it confirmed by a scan, e.g. MRI? Yes ☐ No ☐

15. Does anyone in your close family suffer from tendonitis? Yes ☐ No ☐ Don't know ☐

If yes, which tendon?

*e.g. Achilles*

16. Has anyone in your close family ever ruptured a tendon? Yes ☐ No ☐ Don't know ☐

If yes, which tendon?

*e.g. Achilles*

**PLEASE TURN OVER**



17. Have you ever fully ruptured a ligament? Yes ☐ If yes, which ligament? No ☐  
*e.g. ACL*

18. If Yes, please give details of how this occurred and at what age.

Contact	Non-contact	Age
<i>e.g. tackled from the side</i>	<i>e.g. landing from a jump</i>	<i>e.g. 20</i>

19. Have you ever been told that you have had a ligament sprain/tear? Yes ☐ If yes, which ligament? No ☐  
*e.g. ACL*

20. If Yes, was it confirmed by a scan, e.g. MRI? Yes ☐ No ☐

21. Has anyone in your close family ever ruptured a ligament? Yes ☐ No ☐ Don't know ☐

If yes, which ligament?

*e.g. ACL*



### Bone-Specific Physical Activity Questionnaire (BPAQ)

Participant ID: \_\_\_\_\_

Please list any sports or other physical activities (other than endurance running) you have participated in regularly (e.g. on a weekly basis for a year). Please tick the appropriate age box to indicate how old you were for each sport/activity and how many years you participated for:

[illegible]

# **Genetics of Bone Mineral Density and Stress Fracture Incidence**



## **Menstrual History Questionnaire**

To help complete our research in understanding the possible genetic effects on bone mineral density, could you please answer the following questions. Please be assured that your answers will remain confidential.

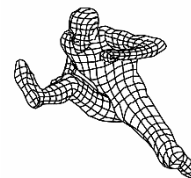
1. At what age approximately did you start your menstrual cycle? \_\_\_\_\_
2. When approximately was your last period? \_\_\_\_\_
3. How often have you had menstrual periods in the last year? (Please circle)  
Once every 20 days or less  
Every 21 – 27 days                      Every 28 – 35 days  
Every 36 – 50 days                      Every 3 – 4 months  
Very irregular (i.e. sometimes monthly, sometimes skip several months)  
Other (Please specify) \_\_\_\_\_
4. Number of menstrual cycles in the last 12 months \_\_\_\_\_
5. My periods usually last \_\_\_\_\_ days
6. What is the longest time you have gone without having a menstrual period? \_\_\_\_\_
7. Have you ever had irregular menstruation? (i.e. spotting between periods/missed periods) YES/NO  
If yes, approximately on how many occasions has this been?  
\_\_\_\_\_
8. Are you on any form of contraception? (Please delete as appropriate) YES/NO  
If yes, please state what type of contraception, if known: \_\_\_\_\_

9. Have you been through, or are you are going through, the menopause? If yes, what age did this occur? \_\_\_\_\_

10. Have you ever been pregnant/had children? If yes, how many times/children?  
\_\_\_\_\_

## **Appendix 2:**

## Department of Exercise and Sport Science



### Informed Consent Form

**(Both the investigator and  
participant should retain a copy of this form)**

Name of Participant:

Principal Investigator: Dr Alun Williams

Project Title: The Genetic Profile of Elite Athletes

Ethics Committee Approval Number: 12.07.11 (i)

### Participant Statement

I have read the participant information sheet for this study and understand what is involved in taking part. Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without giving a reason. Any concerns I have raised regarding this study have been answered and I understand that any further concerns that arise during the time of the study will be addressed by the investigator. I therefore agree to participate in the study.

It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the The University Secretary and Clerk to the Board of Governors, Manchester Metropolitan University, Ormond Building, Manchester, M15 6BX. Tel: 0161 247 3400 who will undertake to investigate my complaint.

Signed (Participant)

Date

Signed (Investigator)

Date



# The Genetic Profile of Elite Athletes Questionnaire: Physical Activity & General Health

Thank you for participating in this research study. We would like you to answer a few questions concerning your general health and physical activity level. Please answer the following questions as honestly as you can.

Participant ID code: \_\_\_\_\_ Date of birth: \_\_\_\_\_

Gender (please tick): Male ☐ / Female ☐ Height: \_\_\_\_\_

Nationality (as on passport, e.g. British): \_\_\_\_\_ Body weight: \_\_\_\_\_

What is your ethnic group? Please tick the appropriate box.

**A) White:** English ☐ Scottish ☐ Welsh ☐ N. Irish ☐ Irish ☐  
French ☐ South African ☐ New Zealander ☐ Australian ☐ Other ☐

If other, please state here: \_\_\_\_\_

**B) Mixed:** White & Black British ☐ White & Black Caribbean ☐ White & Black African ☐ White & Asian ☐ White & Latin American ☐ Other ☐

If other, please state here: \_\_\_\_\_

**C) Asian:** British ☐ Indian ☐ Pakistani ☐ Chinese ☐ Japanese ☐ Other ☐

If other, please state here: \_\_\_\_\_

**D) Black:** British ☐ Caribbean ☐ African ☐ Other ☐

If other, please state here: \_\_\_\_\_

**E) Latin American:** Brazilian ☐ Argentinian ☐ Mexican ☐ Colombian ☐ Other ☐

If other, please state here: \_\_\_\_\_

**F) Pacific Islands:** Samoa ☐ Fiji ☐ Tonga ☐ PNG ☐ Other ☐

If other, please state here: \_\_\_\_\_

**G) Other ethnic background:** ☐ Please state here: \_\_\_\_\_

**I do not wish to state my ethnic origin** ☐

**Using the ethnic groups above as a guide, please tell us the ethnic origin of your:**

Mother: \_\_\_\_\_ Do not know: ☐

Father: \_\_\_\_\_ Do not know: ☐

Mother's mother: \_\_\_\_\_ Do not know: ☐

Mother's father: \_\_\_\_\_ Do not know: ☐

Father's mother: \_\_\_\_\_ Do not know: ☐

Father's father: \_\_\_\_\_ Do not know: ☐

## Blood donation

We would like to take a small (10 mL) blood sample from a vein in your arm. Before doing so, please answer the following safety questions.

1. Have you ever been infected with a blood-borne disease? \_\_\_\_\_ Yes ☐ No ☐
2. Are you anaemic or receiving treatment for anaemia or iron deficiency? \_\_\_\_\_ Yes ☐ No ☐

If you have answered YES to any of these questions and/or you would prefer not to provide a blood sample, a saliva sample may be provided instead.



### Your general health

1. **At present**, do you have any health problem for which you are:
- a) on medication, prescribed (by a doctor) or otherwise \_\_\_\_\_ Yes ☐ No ☐
- b) attending (visiting) your doctor \_\_\_\_\_ Yes ☐ No ☐
- c) on a hospital waiting list \_\_\_\_\_ Yes ☐ No ☐
2. **Have you ever** had any of the following?
- a) Your doctor advised you not to take vigorous exercise \_\_\_\_\_ Yes ☐ No ☐
- b) Pain in your chest when you undertake physical activity? \_\_\_\_\_ Yes ☐ No ☐
- c) Central Nervous System disease, such as Parkinson, Alzheimer, Convulsions/epilepsy \_\_\_\_\_ Yes ☐ No ☐
- d) Have you any history of chest problems, such as bronchitis, asthma or wheezy chest \_\_\_\_\_ Yes ☐ No ☐
- e) Major illness, such as viral hepatitis, cancer \_\_\_\_\_ Yes ☐ No ☐
- f) Eczema \_\_\_\_\_ Yes ☐ No ☐
- g) Diabetes \_\_\_\_\_ Yes ☐ No ☐
- h) High blood pressure \_\_\_\_\_ Yes ☐ No ☐
- i) A limb fracture \_\_\_\_\_ Yes ☐ No ☐
- j) Blood disorder, such as clotting problems, thrombosis, aneurysm, embolus) \_\_\_\_\_ Yes ☐ No ☐
- k) Head injury \_\_\_\_\_ Yes ☐ No ☐
- l) Digestive problems \_\_\_\_\_ Yes ☐ No ☐
- m) Heart problems, such as heart attack, valve disease, palpitations, angina \_\_\_\_\_ Yes ☐ No ☐
- n) Problems with bones, such as osteoporosis or osteoarthritis \_\_\_\_\_ Yes ☐ No ☐
- o) Problems with joints, such as rheumatoid arthritis, any persistent pain, or any surgery on your joints \_\_\_\_\_ Yes ☐ No ☐
- p) Back problems \_\_\_\_\_ Yes ☐ No ☐
- q) Disturbance of balance/co-ordination, such as dizziness or balance-system dysfunction \_\_\_\_\_ Yes ☐ No ☐
- r) Numbness in hands or feet \_\_\_\_\_ Yes ☐ No ☐
- s) Disturbance of vision \_\_\_\_\_ Yes ☐ No ☐
- t) Physical limitations, such as visual, hearing, walking problems \_\_\_\_\_ Yes ☐ No ☐
- u) Thyroid problems, e.g. rapid loss or gain of weight \_\_\_\_\_ Yes ☐ No ☐
- v) Kidney or liver problems \_\_\_\_\_ Yes ☐ No ☐
- w) A severe allergic reaction, e.g. swelling, breathing difficulties in response to an external stimulus \_\_\_\_\_ Yes ☐ No ☐
- x) Emotional or psychiatric problems \_\_\_\_\_ Yes ☐ No ☐
- y) Any other illness or condition that affects your general health or interferes with your daily activities \_\_\_\_\_ Yes ☐ No ☐

4. If you answered **YES** to any of the questions above, please describe the details briefly below or to the investigator if you wish.

\_\_\_\_\_

\_\_\_\_\_

5. Are you currently involved in any other research studies at the University or elsewhere? Yes ☐ No ☐
- If **YES** please provide details of the study:

\_\_\_\_\_

\_\_\_\_\_

## Habitual physical activity

1. What is your main occupation? \_\_\_\_\_
2. At work I sit      Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
3. At work I stand    Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
4. At work I walk      Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
5. At work I lift heavy loads      Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
6. After work I am tired      Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
7. At work I sweat      Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
8. In comparison with others my own age I think my work is:  
     Much heavier ☐    Heavier ☐    As heavy ☐    Lighter ☐    Much lighter ☐
9. Do you play sport or exercise?    Yes ☐    No ☐  
     If **YES**, which sport do you play most frequently? \_\_\_\_\_  
     How many hours per week?      Less than 1 ☐    1 to 2 ☐    2 to 3 ☐    3 to 4 ☐    More than 4 ☐  
     Time per session (hours)      ½ ☐    1 ½ ☐    2 ½ ☐    3 ½ ☐    4 ½ ☐  
     How many months per year?      Less than 1 ☐    1 to 3 ☐    4 to 6 ☐    7 to 9 ☐    More than 9 ☐  
     What proportion of the month?      A few hours ☐    A few days ☐    2 weeks ☐    3 weeks ☐    Most of the month ☐  
     If you do a **second** sport (or exercise class), which is it? \_\_\_\_\_  
     How many hours per week?      Less than 1 ☐    1 to 2 ☐    2 to 3 ☐    3 to 4 ☐    More than 4 ☐  
     Time per session (hours)      ½ ☐    1 ½ ☐    2 ½ ☐    3 ½ ☐    4 ½ ☐  
     How many months per year?      Less than 1 ☐    1 to 3 ☐    4 to 6 ☐    7 to 9 ☐    More than 9 ☐  
     What proportion of the month?      A few hours ☐    A few days ☐    2 weeks ☐    3 weeks ☐    Most of the month ☐
10. Compared with others of my own age I think my physical activity during leisure time is:  
     Much more ☐    More ☐    The same ☐    Less ☐    Much less ☐
11. During leisure time I sweat      Very Often ☐    Often ☐    Sometimes ☐    Seldom ☐    Never ☐
12. During leisure time I play sport      Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
13. During leisure time I watch TV      Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
14. During leisure time I walk      Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
15. During leisure time I cycle      Never ☐    Seldom ☐    Sometimes ☐    Often ☐    Always ☐
16. How many minutes do you walk per day to and from work, school and/or shopping?  
     Less than 5 ☐    5 to 15 ☐    16 to 30 ☐    31 to 45 ☐    More than 45 ☐

**Thank you for completing this questionnaire. All information will be kept strictly confidential.**

# Appendix 3:

**Table 5.1:** Bone phenotype (TBMD, LBMD, LSBMD, T-score and Z-score) means (SD) for each genotype of the 10 investigated variants in males with the associated P-values for the additive model genotype-cohort interaction (G\*C) and genetic association analysis for the main effect of genotype within the total cohort (SNP). N/A and (N/A) indicates that no or only one individual possessed the specific genotype for that SNP.

SNP	Runners					Non-athletes					P - Value	
	TBMD	LBMD	LSBMD	T-S	Z-S	TBMD	LBMD	LSBMD	T-S	Z-S	G*C	SNP
<i>AXIN1</i>												
rs9921222												
CC	1.271	1.465	1.039	0.73	0.68	1.335	1.514	1.262	1.30	1.30	0.619	0.339
	(0.061)	(0.099)	(0.104)	(0.57)	(0.55)	(0.114)	(0.124)	(0.206)	(1.00)	(0.92)	0.459	0.633
CT	1.303	1.492	1.104	1.00	0.98	1.316	1.464	1.182	1.21	1.15	0.189	0.628
	(0.100)	(0.106)	(0.152)	(0.92)	(0.89)	(0.127)	(0.118)	(0.183)	(1.20)	(1.09)	0.761	0.266
TT	1.249	1.448	1.081	0.50	0.49	1.292	1.462	1.132	0.90	0.92	0.602	0.297
	(0.092)	(0.124)	(0.180)	(0.92)	(0.89)	(0.089)	(0.164)	(0.134)	(0.87)	(0.66)		
<i>BDNF-AS</i>												
rs6265												
GG	1.289	1.472	1.095	0.87	0.84	1.319	1.494	1.203	1.24	1.16	0.757	0.791
	(0.095)	(0.108)	(0.144)	(0.88)	(0.84)	(0.901)	(0.110)	(0.168)	(0.87)	(0.76)	0.278	0.572
GA	1.281	1.487	1.077	0.80	0.79	1.301	1.434	1.156	0.94	1.01	0.792	0.638
	(0.098)	(0.115)	(0.169)	(0.93)	(0.92)	(0.156)	(0.164)	(0.211)	(1.50)	(1.27)	0.711	0.632
AA	1.264	1.468	1.066	0.70	0.60	1.400	1.596	1.300	1.90	2.00	0.669	0.772
	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)		
<i>COL1A1</i>												
rs1800012												
AA	1.301	1.501	1.092	0.99	0.97	1.328	1.496	1.200	1.31	1.24	0.668	0.067
	(0.085)	(0.100)	(0.139)	(0.79)	(0.77)	(0.107)	(0.118)	(0.186)	(0.97)	(0.89)	0.178	0.021*
AC	1.259	1.434	1.076	0.59	0.57	1.255	1.406	1.106	0.52	0.65	0.711	0.165
	(0.109)	(0.117)	(0.180)	(1.03)	(0.97)	(0.133)	(0.165)	(0.137)	(1.27)	(1.11)	0.775	0.033*
CC	1.226	1.438	1.151	0.30	0.20	1.360	1.485	1.360	1.53	1.50	0.630	0.066
	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)	(0.093)	(0.126)	(0.149)	(0.81)	(0.70)		
<i>COMT</i>												
rs4680												
GG	1.300	1.516	1.080	0.98	0.96	1.294	1.461	1.127	0.91	0.97	0.432	0.687
	(0.093)	(0.111)	(0.119)	(0.87)	(0.84)	(0.101)	(0.130)	(0.223)	(0.98)	(0.85)	0.581	0.423

GA	1.304	1.489	1.087	1.02	0.99	1.318	1.488	1.201	1.25	1.15	0.735	0.596
	(0.091)	(0.104)	(0.116)	(0.82)	(0.79)	(0.101)	(0.108)	(0.161)	(0.95)	(0.86)	0.536	0.483
AA	1.257	1.444	1.092	0.58	0.55	1.323	1.463	1.211	1.14	1.20	0.393	0.650
	(0.096)	(0.108)	(0.200)	(0.93)	(0.89)	(0.150)	(0.178)	(0.193)	(1.36)	(1.21)		
<i>LRP5</i>												
rs3766228												
GG	1.294	1.480	1.094	0.91	0.88	1.330	1.491	1.211	1.32	1.27	0.521	0.301
	(0.097)	(0.111)	(0.157)	(0.88)	(0.85)	(0.114)	(0.142)	(0.199)	(1.05)	(0.93)	0.366	0.493
GA	1.274	1.478	1.087	0.74	0.72	1.281	1.442	1.141	0.79	0.82	0.391	0.381
	(0.093)	(0.109)	(0.144)	(0.91)	(0.88)	(0.110)	(0.101)	(0.128)	(1.04)	(0.96)	0.397	0.250
AA	1.245	1.397	0.940	0.50	0.50	N/A	N/A	N/A	N/A	N/A	0.470	0.281
	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)							
<i>P2RX7</i>												
rs3751143												
AA	1.289	1.479	1.092	0.88	0.86	1.299	1.458	1.174	1.05	1.01	0.390	0.275
	(0.078)	(0.104)	(0.143)	(0.72)	(0.71)	(0.103)	(0.125)	(0.189)	(0.99)	(0.87)	0.556	0.168
AC	1.267	1.462	1.076	0.64	0.61	1.342	1.501	1.199	1.31	1.30	0.583	0.592
	(0.128)	(0.122)	(0.181)	(1.21)	(1.14)	(0.140)	(0.147)	(0.163)	(1.27)	(1.15)	0.499	0.298
CC	1.408	1.621	1.100	2.00	1.90	1.389	1.602	1.383	1.85	1.85	0.411	0.253
	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)	(0.048)	(0.044)	(0.086)	(0.35)	(0.21)		
<i>TNFRSF11A</i>												
rs3018362												
GG	1.297	1.477	1.110	0.94	0.90	1.358	1.532	1.222	1.48	1.45	0.286	0.043*
	(0.107)	(0.128)	(0.166)	(0.99)	(0.93)	(0.130)	(0.130)	(0.195)	(1.14)	(0.94)	0.057	0.082
GA	1.272	1.480	1.063	0.73	0.73	1.270	1.415	1.156	0.82	0.78	0.753	0.274
	(0.076)	(0.077)	(0.126)	(0.74)	(0.77)	(0.092)	(0.121)	(0.126)	(1.00)	(0.77)	0.468	0.128
AA	1.249	1.464	1.013	0.53	0.47	1.336	1.519	1.204	1.32	1.37	0.312	0.096
	(0.027)	(0.041)	(0.102)	(0.25)	(0.21)	(0.074)	(0.072)	(0.102)	(0.67)	(0.21)		
<i>TNFRSF11B</i>												
rs4355801												
AA	1.294	1.487	1.065	0.94	0.89	1.315	1.473	1.199	1.29	1.15	0.966	0.850
	(0.081)	(0.116)	(0.097)	(0.75)	(0.78)	(0.111)	(0.110)	(0.183)	(1.07)	(0.89)	0.844	0.968
AG	1.276	1.471	1.096	0.75	0.73	1.310	1.487	1.162	1.07	1.08	0.688	0.797
	(0.107)	(0.114)	(0.194)	(1.00)	(0.95)	(0.115)	(0.135)	(0.185)	(1.04)	(1.00)	0.965	0.686
GG	1.294	1.479	1.102	0.92	0.92	1.320	1.466	1.213	1.14	1.17	0.968	0.798
	(0.086)	(0.123)	(0.094)	(0.80)	(0.76)	(0.123)	(0.151)	(0.182)	(1.15)	(0.99)		

VDR

rs2228570

CC	1.296	1.477	1.086	0.94	0.91	1.304	1.462	1.202	1.00	1.01	0.682	0.843
	(0.088)	(0.097)	(0.136)	(0.79)	(0.77)	(0.115)	(0.148)	(0.190)	(1.08)	(0.94)	0.771	0.675
CT	1.269	1.462	1.070	0.68	0.66	1.319	1.483	1.177	1.25	1.20	0.923	0.689
	(0.104)	(0.123)	(0.140)	(1.00)	(0.95)	(0.110)	(0.120)	(0.180)	(1.04)	(0.94)	0.506	0.909
TT	1.299	1.512	1.131	0.99	0.96	1.328	1.494	1.204	1.20	1.17	0.538	0.893
	(0.090)	(0.104)	(0.210)	(0.84)	(0.81)	(0.148)	(0.144)	(0.191)	(1.29)	(1.19)		

WNT16

rs3801387

AA	1.266	1.448	1.043	0.65	0.62	1.342	1.507	1.217	1.34	1.35	0.057	0.730
	(0.088)	(0.107)	(0.128)	(0.84)	(0.81)	(0.123)	(0.122)	(0.206)	(1.09)	(1.00)	0.032*	0.952
AG	1.320	1.519	1.166	1.17	1.14	1.287	1.443	1.157	0.85	0.90	0.042*	0.592
	(0.103)	(0.106)	(0.172)	(0.94)	(0.89)	(0.101)	(0.136)	(0.152)	(0.97)	(0.87)	0.051	0.961
GG	1.270	1.492	1.053	0.73	0.70	1.277	1.442	1.171	1.48	0.80	0.045*	0.731
	(0.066)	(0.084)	(0.037)	(0.61)	(0.62)	(0.979)	(0.161)	(0.149)	(1.30)	(0.81)		

# Appendix 4:



**Table 5.2:** Bone phenotype (TBMD, LBMD, LSBMD, T-score and Z-score) mean (SD) for each genotype of the 10 investigated variants in females with the associated P values for the additive model genotype-cohort interaction (G\*C) and genetic association analysis for the main effect of genotype within the total cohort (SNP). N/A and (N/A) indicates that no or only one individual possessed the specific genotype for that SNP.

SNP	Runners					Non-athletes					P - Value	
	TBMD	LBMD	LSBMD	T-S	Z-S	TBMD	LBMD	LSBMD	T-S	Z-S	G*C	SNP
<i>AXIN1</i>												
rs9921222												
CC	1.200	1.286	1.147	1.10	1.05	1.216	1.249	1.169	1.28	1.40	0.586	0.534
	(0.098)	(0.103)	(0.174)	(1.15)	(1.03)	(0.114)	(0.138)	(0.146)	(1.33)	(1.17)	0.549	0.822
CT	1.196	1.290	1.093	1.08	0.99	1.177	1.218	1.186	0.83	0.79	0.529	0.859
	(0.718)	(0.100)	(0.123)	(0.85)	(0.66)	(0.107)	(0.110)	(0.199)	(1.27)	(1.12)	0.577	0.555
TT	1.223	1.273	1.132	1.40	1.15	1.192	1.262	1.153	1.03	1.05	0.423	0.299
	(0.091)	(0.095)	(0.117)	(1.09)	(0.96)	(0.103)	(0.127)	(0.176)	(1.21)	(1.12)		
<i>BDNF-AS</i>												
rs6265												
GG	1.193	1.276	1.114	1.04	0.97	1.206	1.253	1.210	1.17	1.19	0.219	0.919
	(0.095)	(0.101)	(0.140)	(1.11)	(0.99)	(0.113)	(0.128)	(0.180)	(1.33)	(1.16)	0.189	0.771
GA	1.218	1.305	1.153	1.34	1.18	1.166	1.204	1.124	0.72	0.73	0.091	0.480
	(0.082)	(0.103)	(0.165)	(0.98)	(0.77)	(0.097)	(0.107)	(0.160)	(1.14)	(1.08)	0.212	0.945
AA	1.227	1.282	1.135	1.45	1.30	1.158	1.185	1.031	0.60	0.60	0.199	0.817
	(0.035)	(0.057)	(0.183)	(0.42)	(0.29)	(0.137)	(0.042)	(0.205)	(1.70)	(1.70)		
<i>COL1A1</i>												
rs1800012												
AA	1.191	1.282	1.117	1.01	0.95	1.198	1.239	1.174	1.07	1.08	0.094	0.488
	(0.092)	(0.102)	(0.154)	(1.08)	(0.94)	(0.110)	(0.121)	(0.170)	(1.30)	(1.18)	0.620	0.934
AC	1.240	1.292	1.158	1.60	1.37	1.177	1.226	1.178	0.86	0.89	0.583	0.510
	(0.066)	(0.092)	(0.130)	(0.75)	(0.66)	(0.106)	(0.124)	(0.198)	(1.24)	(1.10)	0.093	0.437
CC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.154	0.590
<i>COMT</i>												
rs4680												
GG	1.218	1.289	1.135	1.33	1.12	1.214	1.251	1.211	1.27	1.33	0.936	0.427
	(0.101)	(0.108)	(0.183)	(1.20)	(1.00)	(0.122)	(0.136)	(0.145)	(1.45)	(1.23)	0.871	0.716

GA	1.196	1.281	1.130	1.07	1.03	1.178	1.220	1.168	0.84	0.88	0.867	0.659
	(0.093)	(0.104)	(0.155)	(1.11)	(0.98)	(0.106)	(0.111)	(0.184)	(1.24)	(1.13)	0.942	0.429
AA	1.198	1.285	1.115	1.11	1.03	1.192	1.246	1.556	1.03	0.98	0.745	0.508
	(0.069)	(0.089)	(0.109)	(0.80)	(0.72)	(0.102)	(0.128)	(0.201)	(1.19)	(1.11)		
<i>LRP5</i>												
rs3766228												
GG	1.217	1.300	1.148	1.32	1.21	1.196	1.230	1.182	1.06	1.06	0.634	0.242
	(0.089)	(0.092)	(0.147)	(1.05)	(0.87)	(0.111)	(0.121)	(0.173)	(1.31)	(1.17)	0.097	0.709
GA	1.167	1.240	1.087	0.74	0.67	1.178	1.253	1.161	0.86	0.94	0.836	0.390
	(0.079)	(0.105)	(0.152)	(0.94)	(0.89)	(0.105)	(0.124)	(0.202)	(1.23)	(1.15)	0.625	0.273
AA	1.235	1.359	1.047	1.55	1.30	1.155	1.149	1.123	0.60	0.50	0.510	0.310
	(0.091)	(0.066)	(0.112)	(1.06)	(0.71)	(N/A)	(N/A)	(N/A)	(N/A)	(N/A)		
<i>P2RX7</i>												
rs3751143												
AA	1.220	1.306	1.137	1.36	1.27	1.207	1.251	1.200	1.19	1.78	0.232	0.030*
	(0.085)	(0.106)	(0.151)	(1.00)	(0.82)	(0.106)	(0.119)	(0.171)	(1.24)	(1.15)	0.601	0.048*
AC	1.159	1.243	1.098	0.64	0.57	1.164	1.208	1.146	0.68(1.	0.72	0.194	0.251
	(0.072)	(0.080)	(0.145)	(0.88)	(0.82)	(0.116)	(0.126)	(0.187)	37)	(1.18)	0.262	0.030*
CC	1.271	1.294	1.187	1.90	1.50	1.129	1.163	0.999	0.30	0.53	0.447	0.022*
	(0.127)	(0.042)	(0.157)	(1.47)	(1.30)	(0.379)	(0.088)	(0.154)	(0.46)	(0.55)		
<i>TNFRSF11A</i>												
rs3018362												
GG	1.216	1.296	1.128	1.32	1.22	1.180	1.224	1.165	0.86	0.93	0.350	0.922
	(0.076)	(0.094)	(0.137)	(0.89)	(0.67)	(0.123)	(0.130)	(0.194)	(1.46)	(1.24)	0.374	0.936
GA	1.200	1.278	1.152	1.11	0.96	1.197	1.237	1.188	1.08	1.06	0.921	0.463
	(0.101)	(0.112)	(0.178)	(1.18)	(1.09)	(0.090)	(0.109)	(0.172)	(1.04)	(1.00)	0.332	0.897
AA	1.158	1.256	1.063	0.61	0.63	1.213	1.286	1.143	1.23	1.18	0.389	0.862
	(0.095)	(0.088)	(0.106)	(1.13)	(1.09)	(0.161)	(0.170)	(0.153)	(1.90)	(1.90)		
<i>TNFRSF11B</i>												
rs4355801												
AA	1.201	1.303	1.131	1.13	1.01	1.238	1.295	1.187	1.58	1.65	0.213	0.391
	(0.083)	(0.110)	(0.155)	(0.97)	(0.92)	(0.093)	(0.108)	(0.144)	(1.06)	(0.92)	0.432	0.070
AG	1.209	1.289	1.123	1.23	1.14	1.170	1.220	1.144	0.75	0.75	0.616	0.509
	(0.084)	(0.096)	(0.153)	(1.00)	(0.82)	(0.096)	(0.109)	(0.171)	(1.13)	(1.06)	0.200	0.360
GG	1.191	1.247	1.128	1.00	0.93	1.196	1.219	1.224	1.04	1.05	0.087	0.237
	(0.110)	(0.122)	(0.140)	(1.28)	(1.08)	(0.132)	(0.142)	(0.209)	(1.56)	(1.31)		

VDR

rs2228570

CC	1.196	1.276	1.107	1.09	1.01	1.173	1.223	1.156	0.79	0.89	0.330	0.134
	(0.074)	(0.090)	(0.107)	(0.87)	(0.81)	(0.094)	(0.106)	(0.186)	(1.11)	(1.04)	0.984	0.405
CT	1.210	1.299	1.147	1.23	1.07	1.219	1.249	1.211	1.34	1.30	0.563	0.190
	(0.098)	(0.105)	(0.183)	(1.15)	(0.97)	(0.114)	(0.133)	(0.171)	(1.32)	(1.17)	0.313	0.124
TT	1.201	1.271	1.125	1.12	1.12	1.134	1.210	1.096	0.31	0.32	0.164	0.200
	(0.101)	(0.109)	(0.152)	(1.20)	(0.98)	(0.098)	(0.113)	(0.174)	(1.16)	(1.04)		

WNT16

rs3801387

AA	1.211	1.299	1.147	1.25	1.14	1.184	1.234	1.183	0.93	0.86	0.658	0.914
	(0.086)	(0.091)	(0.136)	(1.01)	(0.90)	(0.102)	(0.115)	(0.216)	(1.19)	(1.03)	0.555	0.326
AG	1.184	1.245	1.101	0.92	0.84	1.195	1.229	1.179	1.04	1.08	0.814	0.463
	(0.101)	(0.120)	(0.185)	(1.21)	(1.00)	(0.116)	(0.130)	(0.157)	(1.36)	(1.24)	0.659	0.876
GG	1.204	1.304	1.071	1.18	1.13	1.195	1.255	1.145	1.06	1.19	0.472	0.818
	(0.059)	(0.066)	(0.109)	(0.71)	(0.54)	(0.110)	(0.117)	(0.158)	(1.28)	(1.20)		

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